Investigation into the relationships between molecular chaperones, mitochondrial antioxidant enzymes, and endothermic vertebrate longevity

by

Casey Christoff

A thesis submitted to the Department of Biological Sciences

in partial fulfillment of the requirements for the degree

Master's of Biological Science

Faculty Mathematics and Science

Biological Sciences, Brock University

St. Catharines, Ontario

Abstract

The maximum lifespan (MLSP) of endothermic vertebrates can range from as little as a year to over two centuries, yet the underlying phenotype of aging is very similar amongst this group of organisms. One organelle that may be important in the phenotype of aging is the mitochondrion. When damaged, this organelle is thought to contribute to many of the neurodegenerative diseases of aging. For this thesis, mitochondria from brain tissues of 7 mammalian and 2 avian species were isolated to assess whether the antioxidant glutathione system and major molecular chaperone, HSP60, is correlated to species MLSP. Furthermore, HSP60, and the major endoplasmic reticulum chaperone, GRP78, were measured under basal conditions, and following the introduction of an oxidative stress (hydrogen peroxide) in cultured mammalian myoblasts from 10 different species. My results indicate that the enzymes involved in the glutathione defense system are not correlated to species MLSP in brain mitochondria; however HSP60 levels are indeed higher in the longer-lived species. HSP60 levels are also higher at the basal level in cultured mammalian myoblasts and after 1 hour of hydrogen peroxide exposure. GRP78 induction is not correlated to species MLSP at the basal level or following hydrogen peroxide exposure. Therefore, these results suggest that HSP60 is a correlate of longevity in endothermic vertebrate species, but neither the glutathione antioxidant defense system, nor GRP78, correlates to species longevity.

.

Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Jeff Stuart, for if it had not been for his encouragement to embark on a Master's thesis, I might not have gotten to where I am now. His guidance and direction over the past two and half years have helped me discover what I truly am capable of, and that hard work and dedication really does pay off. I would also like to thank my committee members, Dr. Paul Leblanc and Dr. Doug Bruce for their constructive criticisms and guidance during the duration of this thesis, and my external examiner, Dr. Sandra Peters, for her insightful questions and helpful comments.

Many thanks go out to the other members of the Stuart lab. They have not only seen my triumphs, but they have witnessed my failures too. If it had not been for their constant support, encouragement, and coffee breaks, I probably would be even more mental than I am now. Special thanks to Ellen Robb who has been with me every step of the way, celebrating with me when things were good, and offering advice when things went haywire. Her constant encouragement has really made all the difference to my experience here at Brock and I wish her all the best in her new role as a postdoc at Cambridge University.

Lastly, I need to thank my family. When the going got tough, they were there with arms wide open. My mother was always there on the receiving end of a phone call to listen to me when I needed to vent, comfort me when I was feeling overwhelmed, and tell me to get over it when I was blowing things out of proportion. And of course, I would not have made it through this had it not been for my husband, Mark, who has seen me day in a day out throughout this whole experience. He has witnessed firsthand the many faces of Casey, and has stood by me through all of them. We did it babe!

Table of Contents

List of Tables

Chapter 2:

Chapter 3:

Appendix I

Table 5: Non-transformed data for CS induction following H_2O_2 exposure in cultured mammalian myoblasts from 10 different species

Table 6: Non-transformed data for beta actin induction following H_2O_2 exposure in cultured mammalian myoblasts from 10 different species

List of Figures

Chapter 2:

Chapter 3:

Appendix I: Control measurements

Figure 1: MLSP as a function of CS activity in isolated brain mitochondria from 9 endothermic species

Figure 2: MLSP as a function of GPx/GR ratio in isolated brain mitochondria from 9 endothermic vertebrate species

Figure 3: MLSP as a function of body mass for all mammalian and avian species used in this study

Figure 4: Beta actin sequence for all mammalian species from which myoblasts were obtained

Figure 5: MLSP as a function of basal CS activity in cultured mammalian myoblasts

Figure 6: MLSP as a function of beta actin levels in cultured mammalian myoblasts

Figure 7: MLSP as a function of CS activity induction following H_2O_2 exposure

Figure 8: Residual analysis of MLSP as a function of CS activity induction following H_2O_2 exposure

Figure 9: MLSP as a function of beta actin induction following H_2O_2 exposure

Figure 10: Residual analysis of MLSP as a function of beta actin induction following H_2O_2 exposure

Figure 11: MLSP as a function of body mass for all mammalian species used in this study

Appendix II: Human MSLP of 90 years

Figure 1: MLSP as a function of HSP60 levels in cultured mammalian myoblasts after standardization to beta actin and CS activity

Figure 2: MLSP as a function of GRP78 levels in cultured mammalian myoblasts after standardization to beta actin

Figure 3: MLSP as a function of basal CS activity in cultured mammalian myoblasts

Figure 4: MLSP as a function of beta actin levels in cultured mammalian myoblasts

Figure 5: MLSP as a function of CS activity induction following H_2O_2 exposure

Figure 6: Residual analysis of MLSP as a function of CS activity induction following H_2O_2 exposure

Figure 7: MLSP as a function of beta actin induction following H_2O_2 exposure

Chapter 1. Literature Review

1.1 Introduction

It is a common observation that endothermic vertebrates experience two stages of life, a growth and development stage, which ends when the organism reaches sexual maturity, and a senescent/degenerative stage, which ultimately results in diseases, tissue dysfunction, and death. The length and onset of each stage can be vastly different between species. For example, a mouse has a recorded MLSP of 4 years, and reaches sexual maturity 42 days after birth. In contrast, a naked mole rat (*Heterocephalus glaber*), which is similar in size and body mass, reaches sexual maturity 228 days after birth and the species (*Heterocephalus glaber*) MLSP is 31 years. Furthermore, a bowhead whale, reaches sexual maturity only after 22 years and the species' recorded MLSP is 211 years (de Magalhães and Costa, 2009)!

While the above is only one example of how lifespans differ across vertebrate endotherms, there is a vast range of lifespans within this class. However, surprisingly the phenotype of aging is quite similar in all vertebrate endotherms. Many of the major hallmarks of aging (e.g. cataract formation, cancer, heart disease, liver failure, sarcopenia, neurodegenerative diseases, hearing loss) are observed across all endothermic vertebrates, suggesting that aging may look similar at the cellular level in these species (Finch, 1990).

The lifespan of animals depends heavily on environmental factors (predation, disease, and accessibility of nutrients), which all affect the mean lifespan of animal populations. However, researchers studying aging and longevity almost always utilize the species recorded MLSP, which provides useful information on how long an individual of a given species could

potentially live for. This value is most often measured in a sheltered environment absent of predation, malnutrition, and disease (de Magalhães, 2006), and thus provides a measurement of a species' longevity "potential" when external factors that might accelerate the aging rate have been removed. AnAge is an online database containing detailed MLSP data for many animal species from many different sources, including Richard Weigl's mammalian longevity records in captivity (Weighl, 2005). This database assists researchers in ensuring that the value of a species' MLSP is as accurate as possible and that the same values are used across studies (de Magalhães et al., 2005; de Magalhães and Costa, 2009). It is evident from this database that the range of MLSP for all endothermic vertebrate species is quite vast, and yet all individuals in this group experience organismal senescence (aging of the whole organism) resulting in tissue dysfunction and eventually death. This suggests that it is not the actual processes (diseases and tissue degeneration) associated with aging that determine longevity, but rather the timing of the onset of these processes. Therefore, it is hypothesized that longer-lived species are able to prevent the onset of these processes for longer periods of time, thus delaying the aging process and ultimately death.

It should be noted that MLSP data represents a single observation and is not representative of the whole speceis. However, with respect to studies that address longevity, researchers seek to explain how an individual from a given species could reach their longevity potential. Furthermore, it also provides a basis from which all longevity studies can be compared.

1.2 Evolutionary Theories of Aging

Current evolutionary theories of aging predict many different patterns related to life history traits and aging. They combine Charnov's (1993) defined life history traits (adult mortality rate of a species, the age of maturity, and annual fecundity (offspring)) to predict the lifespan of a given species. Charnov (1993) proposed that there is a strong relationship between adult mortality rate and reproduction. Species that experience high predation will have an increased adult mortality rate, resulting in an earlier age of maturity and a higher annual fecundity. Species that experience low predation will have a lower adult mortality rate, a later age of maturity, and a lower annual fecundity (Charnov, 1993). These life history traits are important to three sub theories of the evolutionary theory of aging because they are measurable characteristics that can be interpreted to either support or disprove the hypothesis of increased adult mortality leading to decreased MLSP.

1.2.1 Mutation accumulation theory

The Mutation Accumulation Theory was proposed by Medawar (1952) and suggests that the accumulation of genetic mutations underlies aging. There is weak selection to remove late acting deleterious mutations since there is little or no reproductive cost, so these detrimental mutations will accumulate in old age, and result in organismal senescence. Indeed, naturally occurring mortality from environmental factors, such as predation, will ensure that these late acting mutations are rarely expressed, so there is little opportunity to select for their removal from the genome (Austad, 1993). Assuming this theory to be true, we would expect to see that species experiencing high levels of predation would still display a shorter lifespan if predators

were temporarily removed because of the expression of these late acting deleterious mutations (however, over many generations increased lifespan should evolve). Conversely, species experiencing a lower predation rate would have evolved longer lifespans because natural selection would have had greater opportunity to select against late acting deleterious mutations (Austad, 1993).

1.2.2 Antagonistic pleiotropy theory

A second theory that seeks to explain how aging has evolved is the antagonistic pleiotropy theory proposed by Williams (1957). This theory is very similar to the mutation accumulation theory, but it is based on antagonistically pleiotropic genes, which are genes that have beneficial effects on an individual early in life and deleterious effects later in life. These genes are selected for if they increase an individual's reproductive fitness. Highly predated species are unlikely to survive much past the reproductive stage of their lifecycle. Therefore, any genes that would promote greater fitness in highly predated species would be selected for, regardless of how these genes could affect individuals later in life. One example of an antagonistic pleiotropic gene is *glp-1* which codes for a homologue of Notch that is essential for the proliferation of stem-cells in the germ line (Crittenden et al., 2003). When this gene was mutated in *C. elegans*, it was observed that the mutated individuals produced very few, or no, sperm and eggs, yet they were also longer-lived (Arantes-Oliveira et al., 2002), suggesting that the development of the germ line is detrimental to species lifespan. The selection against pleiotropic genes would result in the removal of these genes, and thus, a decrease in fitness (whether that is lowered annual fecundity, or a later onset of sexual maturation, or both). Essentially, if this theory is true, long lived species would have evolved longer maturation times,

and a delayed age of first reproduction since there is a greater force to remove these antagonistic pleiotropic genes, and also longer life. Indeed, these trends have been observed for many species whose MLSP ranges from days to centuries (de Magalhaes et al., 2009)

1.2.3 Disposable soma theory

The disposable soma theory, proposed by Thomas Kirkwood (1977), posits that the evolution of senescence occurred due to differences in the distribution of effort an individual spends on reproduction vs. cellular maintenance. This theory suggests that each individual of a species has a certain level of intrinsic energy available to maintain both their soma (cellular maintenance), and their reproductive success (Kirkwood et al., 2000). Somatic maintenance ensures that the individual maintains their physiological functions throughout their lifespan, whilst the reproductive maintenance is important for reproductive fitness and species survival. As the individual ages, their soma begins to break down and tissues and organs begin to fail. An individual with more effort directed towards the maintenance of their soma will be able to maintain cellular function longer, delay the onset of tissue failure, and thus live longer. With respect to predation, individuals that experience high levels of predation must allocate more effort to reproduction (thus decreasing the maturation time), rather than somatic maintenance due to the higher probability that the individual will be killed before producing offspring. In this context it would be unproductive for them to allocate more effort to somatic maintenance, since they most likely would not survive long enough for somatic maintenance to be beneficial. It would be better for these highly predated species to dedicate more effort to reproduction to ensure that enough offspring are produced to maintain the survival of the species.

All three theories provide an ecological context for understanding what selection pressures may contribute to the evolution of increasing lifespan. However, they do not shed light on the proximal mechanisms whereby bodily functions and integrity can be maintained for longer durations. These ideas have been explored by cell and molecular biologists, and are outlined in the following sections.

1.3 Longevity is Associated with Stress Resistance

The genetics of longevity began to be systematically explored about 30 years ago in two model organisms, *Drosophila melanogaster* and *Caenorhabditis elegans*, now widely used to study the biology of aging. In 1984, Rose's group demonstrated that ongoing selection for postponed reproduction in *D. melanogaster* produced a population with increased lifespan, but decreased early fitness. In this experiment, two populations of flies were established from the same base population: a control population which was allowed to mate freely (after sexual maturity is reached at 7 days of age), and a population that was created through stepwise delayed mating (experimental group) such that mating was eventually delayed to after 70 days of age. Offspring produced from the experimental group displayed decreased fitness (measured by the total number of eggs produced, and the number of viable eggs produced), but lived longer, than offspring produced from control flies (Rose, 1984). These results demonstrate that longevity is a heritable trait, and that there is a cost to early reproduction since the population that experienced an earlier onset of reproduction (allowed to mate freely) did not live to be as long as the population experiencing a late onset of reproduction. Both the control group and the experimental group were established from the same base population, meaning that the increase in

longevity, and decreased early fitness of the experimental population, was due to some trait(s) inherited from the parental populations from which the experimental group was derived from, but the identity of this trait, or traits, remained unknown.

Following the observations made by Rose (1984) further work was done using the same populations of flies to determine what potential traits could have contributed to the increased longevity of the experimental population. Service and colleagues (1985) showed that the experimental populations, which displayed increased lifespans, were more resistant to many forms of stress, including heat, starvation, desiccation and ethanol vapour 1985). This suggested that increased longevity was associated with stress resistance, yet whether the stress resistance was due to increased longevity, or whether the increased longevity was due to increased stress resistance remained unknown.

Further research aimed to determine the direction of the association between longevity and stress resistance (i.e. causality). Two new populations were created from the experimental group of flies from the original study: one control population and one population subjected to desiccation and starvation events. Surviving flies from the experimental population were then allowed to feed and reproduce, and longevity was determined in offspring from these flies, compared to the control flies. It was discovered that the flies from the experimental populations lived longer than their control counterparts, indicating that selection on stress resistance could lead to increased longevity (Rose et al., 1992). Thus, in *D. melanogaster* longevity can be selected for by mating individuals with greater resistance to various exogenous stressors.

Rose's direct demonstration of selectable longevity and stress resistance sparked interest in determining the particular genes contributing to stress resistance and increased lifespan. Subsequent work in *D. melanogaster* has uncovered two specific genes involved in conferring

longevity, *methuselah* and *chico*. Homozygous mutations of the *methuselah* gene (which encodes a G-protein involved in signal transduction of the insulin like growth factor-1 (IGF-1) pathway and thus the mutation results in decreased IGF-1 signalling (Gimenez et al., 2013)) extends lifespan by approximately 35%, and is associated with resistance to a variety of stressors, including starvation, heat, and dietary paraquat (which induces mitochondrial free radical formation- explained later). 48 hours after 20 mM paraquat treatment, 90% of control flies were dead, compared to 50% of *methuselah* mutant flies. Mutant flies also displayed a 50% increase in average survival time following starvation than their control counterparts. Additionally, mutant flies were able to survive 50% longer under extreme heat (36^0C compared to their physiological temperature of 25^0C) (Lin et al, 1998). Furthermore, mutations in *chico* (which codes for an insulin-receptor substrate, and thus the mutated copy results in decreased IGF-1 signaling), results in a 48% increase in lifespan, and an increase in resistance to starvation (Clancy et al., 2001). Together, these data suggest that stress resistance has a genetic component. When either of the *methuselah* or *chico* genes is mutated, the resultant homozygous mutant individuals are longer lived and show increased resistance to stress. Therefore, these two genes in their wild type form limit the lifespan of *D. melanogaster*, and it does so through decreasing the animals' ability to survive periods of stress. Thus, it can be suggested from these findings that a relationship between stress resistance and longevity does exist in *D. melanogaster*.

A similar heritability of lifespan is also seen in *C. elegans* (Johnson and Wood et al., 1982), and similar to *D. melanogaster*, longevity in *C. elegans* is also associated with an increase in stress resistance. There are many reported mutants of *C. elegans* that demonstrate increased longevity coinciding with enhanced stress resistance, including *age-1*, *daf-2*, *daf-23*, *spe-26*, mutants (Friedman and Johnson, 1988; Larsen, 1993; Vanfletern, 1993; Lithgow et al., 1995;

Kenyon et al., 1993; Murakami and Johnson, 1996). The long-lived mutant, *age-1* (which codes for a phosphatidylinositol-3-OH kinase catalytic subunit and is required for non-dauer formation), was the first so-called gerontogene (gene that affects rate of aging (Johnson and Lithgow, 1992)) discovered in *C. elegans* (Friedman and Johnson, 1988). This particular gene, when mutated, has been shown to increase mean lifespan up to 80% and increase MLSP up to 150%. These long-lived mutants also had decreased hermaphroditic fertility (Friedman and Johnson, 1988). Furthermore, the mutant strains are also more resistant to oxidative stress induced by H_2O_2 (Larsen, 1993), and by paraquat (forms reactive oxygen species (ROS)-discussed later) (Vanfleteren, 1993), as well as to heat (Lithgow et al., 1995), UV light (Murakami and Johnson, 1996), and heavy metal stress including cadmium and copper (Barsyte et al., 2001). Similar results have also been observed for *daf-2* mutants. *daf-2* encodes an insulin/IGF-1 receptor, and is involved in the process of entering the dauer state, which is an alternative developmental stage that this organism can enter when food levels are low. Whilst in this dauer stage, growth is arrested, and reproduction is postponed. Both of these characteristics of the dauer state result in individuals that are developmentally delayed, small, and thin; however, they also have increased stress resistance (Anderson, 1978) and lifespan (Klass and Hirsh, 1976) than their non-dauer counterparts. Interestingly, incorporating the *daf-2* mutation (which results in decreased IGF-1 signalling) after the first larval stage results in a hybrid phenotype wherein individuals become healthy, full-sized adults, yet they live twice as long as the wild-type (Kenyon et al., 1993). Additionally, these mutants are more resistant to heat (Lithgow et al, 199), UV resistant (Murakami and Johnson, 1996), and heavy metals (Barsyte et al., 2001). The same stress resistance has also been shown in mutants of *daf-23* (which is also required for dauer formation), and *spe-26* (which is required for spermatid formation, and

therefore could be another example of an antagonistic pleiotropic gene) (Lithgow et al., 1995; Murakami and Johnson, 1996). Taken together, the above studies on *D. melanogaster* and *C. elegans* demonstrate a heritability component of longevity. Furthermore, this heritable contribution could be due, in part, to increased traits that ameliorate damages caused by both exogenous and endogenous stressors.

1.4 Longevity and Stress Resistance in Mammals

The relationship between longevity and stress resistance has also been studied in mammals, and again, there is some evidence that supports an association between longevity and stress resistance. In mammals the phenotype of aging (degeneration of organ systems, resulting in diseases such as neurodegeneration, sarcopenia, cancer, heart failure, liver failure, cataract formation, bone atrophy, hearing loss, and diabetes mellitus) is broadly similar across the entire class (Finch, 1990). Therefore, it can be suggested that the underlying mechanisms conferring stress resistance in mammals are also quite similar. To study this, mice have been used as the model organism because they are easily maintained in a laboratory setting, and there are long-lived mutant strains of mice that can reveal insights into stress resistance and longevity.

1.4.1 Intra-species comparisons of long-lived mouse strains

1.4.1.1 In vivo studies

As in *D. melanogaster* and *C. elegans*, in certain mutant strains of long-lived mice (e.g. Snell and Ames dwarf mice, and the growth hormone receptor knockout (GHR-KO) mouse) increased lifespan is associated with increased stress resistance at the organism level (Bartke et al., 2001; Bokov et al., 2009; Flurkey et al., 2001; Hauck et al., 2002).

The Snell dwarf mouse, which carries a recessive mutation resulting in hereditary dwarfism was discovered in 1929 by George Snell (Snell, 1929). The Snell dwarf mouse has a mutation in the *pit1* (pituitary transcription factor 1) gene that results in reduced plasma levels of growth hormone (GH), thyroid-stimulation hormone (TSH), and prolactin (PRL) (Li et al., 1990; Theill et al., 1993). The deficiencies of these hormones indirectly reduce the levels of IGF-1 (Hsieh et al., 2002). Together, the decrease in growth hormones combined with the secondary decrease in IGF-1 levels result in an increase in lifespan by up to 50% (Flurkey et al., 2001, 2002). Moreover, Snell dwarf mice also show a lowered activation of a stress response (measured by the activities of key enzymes involved in this response) following an i.p injection (75µg/g body mass) of 3-nitropropionic acid (3-NPA) (which causes free radical generation by inhibiting succinate dehydrogenase (subunit of complex II of the electron transport chain (Huang et al., 2005)). When challenged by 3-NPA, wild type mice displayed increased levels of active enzymes involved in initiating a cellular response to oxidative stress. In contrast, Snell dwarf mice displayed virtually no increase in the activity of any of these proteins (Madsen et al., 2004). These data suggest that Snell dwarf mice are less responsive to stress induced by 3-NPA than their wild type littermates. While it cannot be concluded with full confidence that the lesser response is to increased stress resistance, it can be suggested that stress resistance could play a part in the long life of the Snell dwarf mouse.

Similar to the Snell dwarf mouse, the Ames dwarf also displays reduced levels of GH, TSH, and PRL, increased longevity, and increased stress resistance (Schaible et al., 1961; Sornson et al., 1996; Brown-Borg et al., 1996; Bokov et al., 2009). The Ames dwarf phenotype derives from a mutation in *Prop-1* (prophet of *Pit-1*) which is required for the activation of the *Pit-1* gene (Andersen et al., 1995). Thus, the resulting phenotype is roughly equivalent to that

seen in the Snell dwarf. Lifespan of the Ames dwarf mice is increased by up to 60% compared to normal littermates (Brown-Borg et al., 1996), and there is also evidence that the Ames dwarf mice may also be more resistant to exogenous stressors. When Ames dwarf mice (young adults) were administered a single intraperitoneal (i.p.) injection of 75 mg/kg paraquat (induces oxidative stress via free radical formation), only 20% of them were dead after 5 days of administration compared to 100% of normal controls. Additionally, old dwarf mice displayed greater resistance to paraquat than older control mice, (Bokov et al., 2009). Similar results were observed when Ames dwarf mice and control mice were given an i.p injection of 100mg/kg diaquat, which also induces free radical formation and oxidative stress (Bokov et al., 2009).

In addition to the well documented Snell and Ames dwarf mice, the relationship between longevity and resistance to exogenous and endogenous stressors has also been observed in other long-lived mutant mice. For example, mice homozygous null for the $P66^{SHC}$ gene, a gene involved in regulating the levels of mitochondrial ROS (Trinei et al., 2002), live 30% longer than wild-type mice and are more resistance to oxidative stress induced by paraquat (70 mg/kg i.p.) (Migliaccio et al., 1999). Mice overexpressing the *klotho* gene (which inhibits insulin and insulin-like growth factor-1 signalling) live 20-30% longer (Kuroso et al., 2005), and have a higher survival rate after a 75 mg/kg (i.p.) injection of paraquat (Yamamoto et al., 2006). Taken together, these data further strengthen a role for stress resistance in conferring longevity.

It is important to note, however, that not all data published to date support the correlation between longevity and stress resistance. When Snell dwarf mice were subjected to a 250 mg/kg (i.p) injection of acetaminophen (causes acute liver necrosis), it was observed that the Snell dwarf mice were less resistant to acetaminophen than the wild-type mice (Harper et al, 2007). In the same study, long-lived growth-hormone receptor knockout mice (GHR-KO), which display a

similar phenotype to both the Snell and Ames dwarf mice (Coschigano et al., 2000), were less resistant to liver failure and death than their wild-type littermates. Taken together, the above mentioned data suggest that while there is evidence for increased stress resistance *in vivo* in long-lived mice mutants, it appears to be toxin specific, and thus cannot be confirmed with full certainty that stress resistance alone accounts for the lifespan extension seen in these animals.

1.4.1.2 In vitro studies

One complication of studying mammals *in vivo* is that it generates data that may be difficult to interpret. For example, increased resistance to a toxic heavy metal could be achieved by increasing the activities of one or two organs (e.g. liver or kidneys) to detoxify and/or eliminate that metal, rather than by responses at the level of all individual cells. Understanding what happens at a cellular level offers a different perspective on what processes help ensure longevity, while at the same time avoiding the limitations of using whole animals. One approach to studying processes at a cellular level is by using cultured cells. This approach provides a way of studying the processes underlying aging in a controlled environment that is identical for all cell lines used in the study. The ability to control the environment removes any confounding effects of environment (such as temperature, oxygen and carbon dioxide level, humidity, nutrient source) that could affect the outcome of the experiment. The method of using cultured cells to study stress resistance has been used many times, and has provided a wealth of knowledge on stress resistance.

Most of the research on stress resistance and longevity has been performed on cultured fibroblasts. Generally, dermal fibroblasts from long-lived mutant mouse strains (Ames and Snell dwarf mice) have been shown to be more resistant (measured by cell death) to exogenous

stressors including paraquat, heavy metals, rotenone, H_2O_2, UV irradiation, and heat stress than their non-mutant littermates (Panici et al., 2010; Murakami et al., 2003; Salmon et al., 2005). Similarly, mouse embryonic fibroblasts from $p66^{SHC}$ mutant mice are more resistant to H_2O_2 and UV irradiation (Migliaccio et al., 1999). Furthermore, fibroblasts from Snell dwarf mice are also resistant to oxidative stress induced growth arrest. Generally, cultured cells are grown at atmospheric oxygen (20%), which is approximately 6 times greater than what cells are exposed to *in vivo* (usually 3% oxygen at the cellular level). At such a high oxygen level, mouse fibroblasts can undergo only 8-10 population doublings before cell senescence occurs (Parrinello et al., 2003). However, fibroblasts from Snell dwarf mice can continue to grow in 20% oxygen for much longer without entering senescence. They also have growth rates similar to control cells cultured at 3%, and similar resistance to stress, suggesting that cellular senescence caused by high O_2 exposure is delayed in these cells (Maynard and Miller, 2006). Thus, cultured fibroblasts from long-lived mutant strains of mice are more resistant to many stressors, including oxidative stress. How this observation can be related to the increased longevities of mutant mice will be discussed later (see section *1.4.3*).

1.4.2. Inter-species comparisons of naturally long-lived vs. naturally short-lived mammals

Further support for a relationship between stress resistance and longevity has been provided via inter-species comparisons wherein researchers have evaluated stress resistance in naturally long-lived and naturally short-lived mammals and birds (Kapahi et al., 1999; Harper et al., 2007; 2011; Csiszar et al., 2011). Cultured fibroblasts from a variety of species with varying MLSP have shown that the relationship between stress resistance and longevity seen in *D. melanogaster*, *C. elegans*, and mutant strains of mice, is also observed in naturally long-lived vs. naturally short-lived species. The comparative approach to studying aging and longevity allows

us to gain an unbiased understanding of what traits have been selected for during the evolution of longevity, and why a vast range of lifespans in endothermic vertebrates is observed.

Using a similar approach seen in the studies with mutant mice, Kapahi et al., 1999, treated cultured fibroblasts from naturally long-lived and naturally-short lived mammalian species (8 species in total: rat, hamster, marmoset, rabbit, sheep, pig, cow, and human) with paraquat, H_2O_2, sodium aresenite, and sodium hydroxide (using various concentrations to obtain a dose-response relationship). They measured the lethal dose (LD_{90}) that resulted in 90% decrease in DNA synthesis (a proxy of cell death, or at least failure to replicate) after treatment with these stressors, and observed that the long-lived species were able to maintain cell viability after higher doses of these toxins than the shorter-lived species used in this study. These results demonstrated that there was a significant positive correlation between MSLP and resistance to all stressors tested (Kapahi et al., 1999). A similar relationship between stress resistance and longevity was observed in cultured fibroblasts from eight different rodents and the little brown bat with varying lifespans (ranging from a MLSP of 2.7 years to 34 years) (Harper et al., 2007). Treating cultured fibroblasts from these 9 species with cadmium, H_2O_2, heat, and rotenone, and calculating the LD_{50} (the dosage of toxin that resulted in 50% cell death), resulted in positive correlation between stress resistance and lifespan (Harper et al., 2007). Furthermore, when analyzing this relationship in primate fibroblasts (14 different species with MLSPs that vary from 20.5 years to 90 years), the longer living primates displayed a significant decrease in cellular apoptosis following H_2O_2 exposure (300 μmol/L), and thus increased resistance to oxidative stress (Csiszar et al., 2011).

The role for resistance to stress in establishing longevity has also been evaluated in non-mammalian fibroblasts (Harper et al., 2011). Fibroblasts from multiple bird species (35 species

in total with MSLPs ranging from 5 years to 50 years) were treated with cadmium, H_2O_2, paraquat, and methyl methanesulfonate (which induces DNA damage). The LD_{50}'s for the longer living avian species were significantly higher than the shorter lived avian species, indicating that they too were more resistant to cellular stress (Harper et al., 2011). Taken together, the above mentioned studies reveal an association between longevity and stress resistance. However, some results from these studies refute this relationship. For example, some groups have shown that fibroblasts from longer-lived species are more resistant to paraquat treatments when compared to shorter lived species (Kapahi et al., 1999), while other groups have not found this to be true (Harper et al., 2007). Additionally, some stressors do not display a relationship between lifespan and stress resistance, such as UV light and various inducers of the unfolded protein response (discussed later) (Harper et al., 2007; Harper et al., 2011). Collectively, these results suggest that there may be a correlation between stress resistance and lifespan, but this correlation is highly dependent on the type of stress and damages incurred as a result of the stress. Furthermore, these data fail to provide any insights into the mechanisms which could contribute to the increased stress resistance observed in long-lived animals.

1.5 Molecular Mechanisms of Stress Resistance and Longevity

It has been demonstrated from early work (e.g. genetic studies on *D. melanogaster* and *C. elegans*) that longevity has a heritable component. Evolutionary biologists aim to explain why there are such vast differences in lifespan, whereas cellular biologists aim to explain how cells have adapted to ensure longevity in long-lived species. What are the molecular mechanisms that enable such differences in lifespan across the animal kingdom? How can all animal cells be so

similar, yet lifespans so different? These are the questions that researchers in biogerentology are trying to answer.

1.5.1 Cellular damage and longevity

As previously discussed, there appears to be a relationship between stress resistance and longevity across a wide range of species. (Rose, 1984, Service et al., 1984, Lin et al, 1998; Johnson and Wood, 1982, Kenyon et al., 1993, Lithgow et al., 1994, Salmon et al., 2005; Kapahi et al, 1999; Harper et al., 2007, 2011; Csiszar et al., 2011). This seems intuitive: in people we can observe effects of excessive exposure to exogenous stressors on the aging phenotype (e.g. UV stress and skin aging). Understanding how (and which) stresses can lead to an earlier onset of aging can help researchers determine cellular target that might confer longevity in longer-lived species, and ultimately help humans stay "younger" longer by delaying the onset of age-related diseases. Cellular aging is the physiological decline in cellular function, resulting in a complete loss of a cell's replicative ability (cell senescence), loss of normal functions, and even cell death. This can lead to degeneration of whole tissues due to the inability to replace damaged or dead cells. In turn, this degeneration appears to underlie the loss of tissue function in many organs associated with age, and ultimately, death of the organism (organismal senescence). When damage to mitotic cells from exogenous and endogenous stresses becomes too great, cells will enter a senescent state wherein they stop replicating indefinitely (Campisi, 2001). This state helps prevent the spread of the accumulated damage to the next generation of cells, which is beneficial because it stops damages from replicating and accumulating. Senescent cells have also been recognized as a way for cells to prevent the incidence of cancer, since tumorigenesis requires the ability to proliferate in order for its development (Campisi, 2001). However,

senescence also hinders the ability for regeneration. Terminally differentiated cells are non-replicative, but they retain the ability to re-enter the growth phase upon activation by the right growth factors. Therefore, when damage accumulates, the cells will initiate the apoptosis cascade resulting in cell death. Therefore, senescent cells may prevent the spread of damages by the inability to replicate, but this inability also contributes to tissue degeneration due to the fact that they are unable to replace any dead terminally differentiated cells. To put this into context, when terminally differentiated muscle cells (e.g. myotubes) become damaged and cells start to die, they will need to be replaced. However, if the precursor cells (e.g. myoblasts) are in a senescent state, then they will be unable to replace the dead cells, and thus the tissue will continue to degenerate.

In addition to the above mentioned consequences of senescence, senescent cells can also affect neighbouring cells through the secretion of specific soluble signalling factors from senescent cells (Campisi, 2001). The 'senescent cell secretory phenotype' has been described in cultured fibroblasts, wherein senescent fibroblasts secrete a variety of factors that influence the niche (including other cells) around them. These released factors can activate many cell-surface receptors of nearby cells (in tissues for example), initiating signal transduction pathways that may lead to cellular damage, and ultimately cell death (for a detailed review see Coppe et al., 2010). Death of the neighbouring cells, combined with an inability of the tissue's resident stem/progenitor cells to replicate and replace the damaged cells, can result in tissue failure and ultimately organism death. It therefore follows that species in which fibroblasts are more resistant to stress could postpone the onset of this secretory phenotype and in turn delay the onset of niche deterioration that might elicit the death of neighbouring tissue cells and tissue failure.

Many post-mitotic and terminally differentiated cells (such as neurons) lose their replicative ability early in adulthood, yet most species live long after early adulthood. In addition, post-mitotic cells will eventually die due to the accumulation of damages, and this death leads to the onset of many diseases of aging, such as neurodegenerative disease and muscle degeneration. Therefore, species that are longer-lived might have some method of rectifying cellular damages caused by endogenous and exogenous stress so that these post-mitotic cells can continue to function for longer periods of time before dying.

At the cellular level, oxidative insults to macromolecules can cause a wide array of responses, including reduced proliferation, senescence, growth arrest, or apoptosis. In addition, these responses differ based on cell type and the agent (and dosage) used to induce oxidative stress (Martindale, 2002). While aging is thought to occur due to a combination of each of these processes, it is important to understand how cellular damage can lead to cell death as this process is essential to tissue degeneration, which ultimately results in the death of an individual. While there are multiple apoptotic signals, there are just two main pathways by which apoptosis occurs: the extrinsic and the intrinsic pathway. The extrinsic pathway is initiated when the cells receive a death-triggering signal (such as tumor necrosis factor) that binds to death receptors located on the cell membrane. Once bound, a signalling cascade is started that results in caspase activation and apoptosis (Algeciras-Shimnich et al., 2002.). Initiation of the extrinsic pathway also involves the second pathway, the intrinsic or mitochondrial pathway of apoptosis. When the intrinsic pathway is initiated, pro-apoptotic factors are released from the mitochondrial intermembrane space, and these activate caspases and signal the cell to become apoptotic (Mignotte et al., 1998; for a recent review see Wyllie, 2010). Therefore, species that are better at preventing damage to macromolecules (or effectively repair the damage before apoptosis is induced) caused by

endogenous and exogenous stress may be able to delay the initiation of apoptosis, thus reducing the number of cells dying. Furthermore, they may be able to delay cells entering a senescent state. As a consequence, these species will lose cells at a reduced rate and maintain a replicative replacement population longer. Together, this could postpone the onset of tissue dysfunction and delay the onset of an aged phenotype, resulting in a higher MLSP.

1.5.2 Mitochondria, reactive oxygen species (ROS) and apoptosis

Harman's free radical theory of aging (Harman, 1956) suggests that oxidative damage caused by free radicals contributes to the rate of aging. In 1972, Harman modified his theory, implicating mitochondria as the source of intracellular free radicals. During respiration, electrons leak from respiratory complexes of the electron transport chain and reduce molecular oxygen to produce the superoxide anion (reviewed in Murphy, 2009). This molecule can be highly toxic, based on the strong phenotype associated with manganese superoxide dismutase (MnSOD) knockout mice. These mice display extensive mitochondrial injury, degeneration of neurons, and progressive motor disturbances (Lebovitz, 1996). Elevated superoxide levels can cause substantial damage to the abundant iron-sulfur containing proteins found in mitochondria. Iron/sulfur containing proteins are essential to many reduction oxidation reactions that occur in mitochondria and by inactivating these enzymes (by damaging them), superoxide can have strong negative effects on energy production which may underlie the toxicity of superoxide. In addition to the damaging effects of superoxide on iron sulfur clusters in mitochondria, it can also enter into other reactions that result in other toxic agents. For example, dismutation of superoxide gives rise to H_2O_2 via two superoxide dismutases (copper zinc SOD found in the cytosol, and MnSOD found in mitochondria). Furthermore, superoxide can also react with nitric

oxide to produce peroxynitrite, which is also very toxic and highly reactive (Packer et al., 1996). Together, these forms of ROS can cause a multitude of damages to cellular macromolecules, leading to induction of apoptosis, or cell senescence.

There is a strong connection between ROS and the mitochondrial pathway of apoptosis. High ROS levels promote the peroxidation of cardiolipin and affect its interaction with cytochrome c, which is located on the inner mitochondrial membrane (Ott et al., 2002). Cytochrome c is then able to leave the mitochondria through specific channels that form during the apoptotic process. There are two main channels involved in mitochondrial initiation of apoptosis. Opening of the permeability transition pore (stimulated by high oxidant concentration) results in cellular water moving into mitochondria causing membrane swelling and rupturing which allows pro-apoptotic factors to initiate the apoptotic signalling cascade in the cytosol. The other channel, mitochondrial apoptosis channel (MAC) is opened during times of oxidative stress due to an increase in ROS which results an in increase in pro-apoptotic Bcl-2 family members (Bax and Bak, which open the channel) and decreased levels of Bcl-2 (an anti-apoptotic Bcl-2 family member, which closes the channel). Once cytochrome c is released from either channel, it binds various apoptotic factors forming the apoptosome, which activates caspase-3 resulting in cell death (Mignotte and Vayssiere, 1998). Taken together, these data suggest that ROS can initiate the apoptotic pathway via the intrinsic mitochondrial pathway. Therefore, it seems logical that longer-lived species could either produce less ROS, thus preventing damages from happening, and/or have better mechanisms involved in removing ROS and repairing damages to macromolecules caused by ROS which would result in lower accumulated levels of these damages. This decrease in damages could result in a delayed onset of apoptosis, cell senescence, and an aged phenotype, resulting in an increased MLSP.

1.5.3 ROS production and MLSP

In general, larger animals live longer and have a lower metabolic rate than smaller animals (Speakman, 2005). This finding can be explained by the rate of living theory proposed by Pearl in 1928. This theory formulates the hypothesis that there is a fixed amount of metabolic potential for each species, and as such, species that use up their metabolic potential faster will experience accelerated aging (Pearl, 1928). However, if this was indeed an explanation for why species age at different rates, then we would expect to see higher levels of ROS production in shorter lived species since they have an increased metabolic rate. Nevertheless, evidence from the literature is non-conclusive on whether shorter-lived species produce more. While some researchers have revealed a negative correlation between mitochondrial ROS production and species MLSP (Sohal et al., 1990b; Sohal et al., 1993; Ku et al., 1993; Herrero and Barja, 1998), other researchers found no difference in ROS production between long-lived and short-lived species (Lambert et al., 2007). Furthermore, data from unusually long lived animals for their body size (birds, bats, and naked mole rats) also fail to demonstrate a correlation between ROS production and MLSP (reviewed in Robb et al. 2014). Together, these data suggest that ROS may be involved; however the data is ambiguous, and as such, do not provide a clear picture on whether longer-lived species produce less ROS.

1.6 Mitochondria in Diseases of Aging

Mitochondria have often been the focus of aging research due to their involvement in any of the diseases related to aging (neurodegenerative diseases, sarcopenia, cancer and cardiovascular disease for example) (Lin et al.,2006; Schapira et al., 2006; Gogvadze et al.,

2008; Marzetti et al., 2010; Fulle et al., 2004; Ballinger, 2005). Many of these diseases are associated with accumulation of damaged macromolecules. For example, Parkinson's disease (which is disease associated with aging resulting in dysregulated motor movement (tremors) and a decrease in motor ability) results from an increased incidence of cell death in the substantia nigra region of the brain, as well as an increase in the formation of protein aggregates composed of α-synuclein (Baba et al., 1998) and ubiquitin (Betarbet et al., 2001). Additionally, patients with Parkinson's disease also display decreased complex 1 activity, which results in increased ROS formation (Schapira et al., 1990). Furthermore, researchers have induced Parkinson's disease in rats using MPTP (1-methyl-4-phyenyl-1,2,3,6-tetrahydropyridine) and rotenone, both of which are toxins that cause inhibition of complex 1, and thus increased ROS formation (Cannon et al., 2009; Ohashi et al., 2006; Rojo et al., 2005). Together, these data suggest that Parkinson's disease can be a result of mitochondrial dysfunction that results in increased ROS formation, increased DNA mutations, and increased protein damage.

Alzheimer's disease is another neurodegenerative disease that results from improperly functioning mitochondria and oxidative damage to macromolecules. This disease is characterized by a decrease in cognitive function and the formation of protein aggregates composed primarily of amyloid-β peptide (Lin et al., 2006). Damage to the protein complement of Alzheimer's patients could be due in part to oxidative damage. Oxidative damage has been shown to occur before the onset of Alzheimer's disease pathology in the human brain (Nunomura et al., 2001; Sultana et al., 2013) as well as in a mouse model of Alzheimer's disease, which displayed increased lipid peroxidation preceding amyloid- β peptide aggregate formation (Practico et al., 2001). Moreover, evidence suggests that Alzheimer' patients also exhibit increased levels of

mtDNA mutations, increased levels of lipid peroxidation, and increased levels of protein oxidation (for a detailed review see Radak et al., 2011).

Dysfunctional mitochondria have also been observed in other degenerative diseases associated with aging. Sarcopenia is a degenerative disease of aging resulting in a decrease in muscle mass and strength due to an acceleration of apoptosis in aged muscle cells (Dirks et al., 2002; Marzetti et al., 2008). Researchers have found elevated levels of pro-apoptotic factors, Bax and Bak, in aged muscles with a respective decrease in anti-apoptotic factor Bcl, suggesting that mitochondria may be involved in inducing age-related apoptosis of muscle cells (Alway et al., 2002; Song et al., 2006). Furthermore, electron transport chain abnormalities (deficient cytochrome c oxidase activity) are elevated in patients with sarcopenia (Bua et al., 2002). Collectively, these data suggest that the accumulation of mitochondrial abnormalities may contribute to the onset of sarcopenia.

Muscle cells have a capacity to replace damaged and dead cells. Muscle specific stem cells, called satellite cells, reside between the differentiated contractile myofibres. These satellite cells can be stimulated in response to muscle injury (in the case of sarcopenia this would be a decrease in muscle mass) to divide asymmetrically to give rise to muscle-precursor cells called myoblasts. Myoblasts are committed to becoming skeletal muscle (Tedesco et al., 2010). Degeneration of muscle tissues also occurs as an individual ages. There are two possible roles that satellite cells may play in aging. First, a larger stem cell pool would allow for more muscle regeneration therefore maintaining normal muscle function for longer; however, there is no evidence for this possibility. Secondly, satellite cells that are more resistant to stress might be able to retain their function for longer periods of time, thus maintaining regenerative capacity in muscle over longer periods of time. With respect to this second possibility, it is important to note

that other cell types (e.g. fibroblasts) from longer lived animal strains or species are more resistant to stressors and appear to retain their normal function for longer (Harper et al., 2007;2011; Csiszar et al., 2011).

1.7 Mitochondrial Antioxidant Defense Systems and Longevity

Superoxide produced from the electron transport chain is removed from the mitochondrial matrix via MnSOD. MnSOD is a mitochondrial antioxidant enzyme that removes superoxide from the mitochondria by converting it to H_2O_2 which may diffuse from the mitochondria and can be removed from the cell via further enzymatic reactions. Superoxide can spontaneously dismutate into H_2O_2, however MnSOD allows for a more rapid elimination of the superoxide anion (approximately a 10-fold faster rate) (Fridovich, 1983). There is an extensive literature demonstrating how internal stresses (such as the superoxide anion) can cause damage to cellular macromolecules and how the accumulation of this damage can lead to senescence, apoptosis, and ultimately death of an individual (for a detailed review see Kregel et al., 2007). To help protect against internal stresses, such as ROS, cells possess an array of antioxidant enzymes that defend against ROS. Antioxidant enzymes are a cells' first line of defence against ROS by transforming it to less reactive substances.

SODs are responsible for removing superoxide from the cellular environment and have been shown to play a role in the aging process. CuZnSOD homozygous knockout mice have high levels of oxidative damage and show accelerated aging, as well as a decrease in lifespan by approximately 30% (reviewed in Muller et al., 2007). In contrast, MnSOD homozygous knockout mice die very soon after birth (Li et al., 1995), but, heterozygotes are completely viable

and experience lifespans similar to wild type mice. This normal lifespan in heterozygote MnSOD mice is interesting because they not only possess less MnSOD , but they also show no increase in the levels of other antioxidant enzymes (including GPx, CuZnSOD and catalase) to compensate for the lower levels of MnSOD (Van Remmen et al., 1999). However, heterozygotes are more sensitive to paraquat treatments (Tsam. et al., 1998), and have higher levels of oxidative damage. They also show no difference in the incidence and timing of many various aging biomarkers when compared to their wild-type littermates (Van Remmen et al., 2003). In contrast, flies that overexpress MnSOD show increased longevity, approximately 16% longer lifespan (Sun et al., 2002). Taken together, these findings do confirm a role for MnSOD in conferring longevity.

Genetic manipulations of particular genes that are thought to confer longevity in species provide the foundation for comparative analyses. Evidence suggests that SODs may be important to species longevity, and if this relationship is true in all species, then longer-lived species should express higher levels of SODs. The Snell dwarf mice are much longer lived than their wild-type littermates, so it seems logical to predict that they may have higher activity levels of both CuZnSOD and MnSOD. However, it was shown that the Snell dwarf mice showed no differences in CuZnSOD levels and lower levels of MnSOD in heart and brain tissue when compared to normal littermates (Page et al., 2010). Furthermore, a broad comparison analysis, with approximately 15 different species, was utilized to compare levels of CuZnSOD and MnSOD in liver, heart, and brain tissue. From this research it was determined that only MnSOD levels had a positive correlation with MLSP and this was only seen in brain tissue (which remained after being corrected for body mass and phylogenetic independence), but not in any other tissue type studied (heart and liver) (Page et al., 2010). This same study showed that CuZnSOD did not correlate with species MLSP in any tissues, suggesting its lack of involvement

in conferring longevity (Page et al., 2010). The literature provides an abundance of equivocal evidence for CuZnSOD and MnSOD as a critical enzyme to stress resistance, and perhaps, conferring species longevity, suggesting that perhaps superoxide production is not responsible for differences in lifespan.

SODs are only one of many important antioxidant enzymes that are readily available to remove ROS from the cell. The product of the SOD catalyzed reactions, H_2O_2, is still very toxic to the cell. This organic compound readily reacts with ferrous iron (II) causing its oxidation into ferric iron (III). A by-product of this oxidation is the hydroxyl radical which is highly reactive and will react with proteins and lipids quite easily causing their oxidation and subsequent loss of function. Lipid peroxidation is very dangerous as it can result in cell death. Recall, cardiolipin is associated with cytochrome c in the mitochondria. When cardiolipin becomes oxidized, cytochrome c is released from it and is free to leave the mitochondria and initiate the signalling cascade for apoptosis. To help protect against the hydroxyl radicals, cells have developed a system that removes H_2O_2, and other hydroperoxides, from the cell environment which helps minimize the amount of hydroxyl radicals formed.

One system involved in hydroperoxide removal is the GSH system. This system is highly conserved amongst the animal kingdom, as it is present in yeast, nematodes, insects, and mammals. It is composed of many different proteins that work together to maintain the cellular redox state. GSH is a tripeptide that has oxidizing and reducing capacity. It can reduce cellular disulfide bonds that form during times of stress, and it acts as an electron donor for GPx which is the enzyme directly involved in H_2O_2 dismutation into water. Oxidized glutathione (GSSG) is rendered useless, so it must be restored to its active reduced form. This reduction is achieved by

GR. Together, these proteins form the GSH system (see Meister and Anderson, (1983) for a full review on GSH).

GSH is the major thiol in cells and its ability to detoxify hydroperoxides is completely dependent on its reduced state, however GSH cannot reduce hydroperoxides by itself. It is dependent on GPx to reduce the hydroperoxides to water. In addition, GR is critical in maintaining a reduced GSH pool. It therefore follows that these two enzymes crucial for ROS detoxification and that species with higher MLSP might have higher levels/activities of these enzymes than shorter-lived species. When GPx4 (a mitochondrial isoform of GPx and the only isoform capable of removing hydroperoxides from lipids) was overexpressed in hearts of mice, it was found that these mice had much lower levels of lipid peroxidation following ischemic/reperfusion events than control mice (Dabkowski et al., 2008). Additionally, mice overexpressing GPx1 (the most abundant isoform) showed increased resistance to paraquat and diaqaut (Lie and Cheng, 2005), and overexpression of GPx4 prevents oxidative stress-induced apoptosis in mice (Ran et al., 2006). Similarly, overexpression of GR increases lifespan in *D. melanogaster* under hypoxic conditions (Mockette et al., 1999). This increased resistance to stress by overexpression of GPx and GR suggests that longer-lived species may also have higher levels of these two enzymes. However, no correlation between MLSP and activities were found when multiple mammalian and avian species were analyzed for GPx and GR activities, or when Snell dwarf mice and normal littermates were compared for GPx and GR activity (Page et al., 2010; Sohal et al., 1990a). Therefore, further studies into whether GPx and GR are important in conferring longevity need to be performed.

1.8 Protein Homeostasis and Longevity

Protein homeostasis is essential to cellular stress resistance. Stress causes damage to the proteins resulting in altered structure and function of the protein, which further leads to protein aggregation. Many neurodegenerative diseases of aging are associated with protein aggregation (Baba et al., 1998). The incidence of neurodegenerative diseases increases with age, it therefore follows that individuals with better protein homeostasis mechanisms may be better equipped to repair the damages to proteins, delaying their aggregation. Additionally, the unfolded protein response (UPR) is a very quick response to unfolded proteins that is present in all organisms. This response is also present in multiple cellular compartments (endoplasmic reticulum and mitochondria), further supporting the importance of protein homeostasis during times of cellular stress. Recent studies have also shown that the UPR response decreases with age, which may contribute to more protein damage and aggregation, resulting in an aged phenotype and many age related diseases (Sherman M.Y., et al., 2001; Winklhofer K.F., et al., 2008). It therefore follows that species with better protein homeostatic mechanisms could delay the decline in the UPR response, and ultimately delay the onset of an aged phenotype.

The key effectors that elicit the UPR are molecular chaperones. Molecular chaperones, or heat shock proteins (HSPs), are both constitutively expressed, as well as stressed induced by heat, oxidative stress, UV light, and other stresses. They have also been shown to be involved in the aging process. *C. elegans* with reduced expression of HSP70 show advanced aging (Kimura et al., 2007). Moreover, overexpression of HSP70 in mice rescued premature aging and delayed death in these individuals (McArdle et al., 2004; McLean et al., 2002), and overexpression of HSP22 in *D. melanogaster* also resulted in an increase in longevity of approximately 30%

(Morrow et al., 2010). These findings are further strengthened by the discovery of decreased protein mis-folding during stress in the long-lived naked mole rat. The naked mole rat is a very unique species because they live approximately three times longer than similarly sized mice, yet they have similar levels of oxidative damage (Andziak et al., 2006). One group has discovered that proteins from naked-mole rats are more resistant to protein misfolding during times of stress, and have more rapid removal of oxidized proteins (determined by level of protein ubiquitination which was increased in aged mice but not in aged naked mole rats) (Perez et al., 2009a). These findings suggest that molecular chaperones, and protein homeostasis as a whole, are involved in conferring longevity in species.

While the above experiments establish a role for molecular chaperones in longevity, they fail to elucidate whether molecular chaperones contribute to the naturally long lifespan of some species. To study this, a comparative approach should be taken which includes naturally long and naturally short lived species. With this approach, the levels of molecular chaperons, as they are naturally expressed, can be measured and compared to reveal if a positive correlation between molecular chaperones and lifespan exists. This approach was used in a study that measured HSP70 levels in whole liver tissue from the long-lived naked mole rat and short-lived laboratory mice. It was found that the levels of HSP70 were not different between the two included species (Perez, 2009a), however it cannot be concluded from the results of this study that HSPs are not higher in long-lived species due to the lack of statistical power in a two species comparison. In order to determine a relationship with statistical power, more species must be included. Salway et al. (2011a) performed a broad species comparison which measured four molecular chaperones (HSP60 (mitochondrial), HSP70 (cytosolic), GRP78 (endoplasmic reticulum), and GRP94 (endoplasmic reticulum)) in three different tissues (brain, heart, and liver) from 15 endothermic

vertebrate species. It was determined that the levels of all four molecular chaperones were positively correlated with species MLSP in all three tissues (Salway et al., 2011a).

1.9 Myoblasts: A New Model System for Studying Aging

As mentioned above, a bulk of evidence demonstrating a role for stress resistance and longevity comes from studies involving cultured fibroblasts (Kapahi et al., 1999; Harper et al., 2007; 2011; Csiszar et al., 2011). Fibroblasts are precursor cells that are required to maintain the structural integrity of connective tissue by secreting the necessary components of the extracellular matrix. They are a good model for studying aging because overtime they will become damaged and senesce (as seen in the epidermis of aged individuals). It is hypothesized that when fibroblasts senesce they secrete a multitude of molecules that can affect the niche of the surrounding cells, and thus initiate the neighbouring cells to senesce as well (Coppe et al., 2010). This property of fibroblasts makes them a good choice for a cellular model of aging, however it is important not to extrapolate too much from fibroblasts since they cannot be representative of all the various cell types found in endothermic vertebrates.

One cell type that makes a good candidate for studying aging is myoblasts. Myoblasts are muscle precursor cells that are responsible for the repair and maintenance of skeletal muscle (Tedesco et al., 2010), and they can be easily extracted from muscle biopsies and grown in cultured conditions (Rosenblatt et al., 1995). In addition, myoblasts offer a unique cell type to study stress resistance and longevity because they have the ability to differentiate into post-mitotic cells (myotubes) which resemble functional muscle fibres. Myotubes have abundant and highly organized actin and myosin similar to muscle fibres, they have the ability to contract

spontaneously in culture, and they also have high levels of oxidative phosphorylation (e.g. Robb et al., 2012). As individuals age they can experience a decline in muscle mass by up to 40% and a loss of muscle strength by approximately 30% (Moulais et al., 1999). This decrease in muscle mass and strength, combined with a decreased replicative ability of muscles, are the characteristics associated with sarcopenia (a disease associated with old age). Therefore, the ability to maintain a functional myoblast population is important in ensuring muscle mass repair, and as such, myoblasts from longer-lived species may be more stress resistant than those from shorter-lived species.

1.10 Hypotheses and Approaches

1.10.1. GPx, GR, and HSP60 in isolated mitochondria from brain tissue of naturally long- and short-lived species.

Based on the background discussed above, three main, testable ideas can be proposed. First, there is a general relationship between cellular stress resistance and longevity in animals. Second, brain mitochondria are thought to be implicated in many of the diseases associated with aging (all of which are associated with macromolecular damage). Third, there appears to be a role for antioxidant enzymes and chaperone proteins in conferring resistance to stress.

A major limitation with the evidence supporting a role for antioxidant enzymes and chaperone proteins in longevity is that the measurements made have been in whole cells, with both cytosolic and mitochondrial isoforms of the enzymes present. Therefore, in order to understand what is happening in mitochondria specifically, they must be isolated. For the purpose of this thesis, mitochondria were isolated from brain tissue of naturally long-living and

naturally short-living species (7 mammalian and 2 avian species) and the activities of GPx (and GR) as well as the levels mitochondrial HSP60 were measured. With these isolated mitochondria from nine species I tested the hypothesis that the activities of GPx and GR (measured spectrophotometrically), and the levels of HSP60 (measured by Western blot) would be higher in longer-lived species.

1.10.2. Stress-induced response in cultured myoblasts from naturally long- and short-lived species.

A limitation with looking at stress-resistance in whole tissues is that tissues have different populations of cell types, and the proportions of these cell types may differ from species to species. In addition, the tissues are excised from individuals of a species that may be exposed to different variables that are uncontrolled (e.g. diet, temperature, stresses, inbreeding). It is also difficult to impose a stress at the level of the whole tissue, so conclusions are mostly made based on basal levels of proteins. There could be a potential role for a species' ability to induce a stress response, and that this induction could happen quicker, or be of greater magnitude in long-lived species.

To address these limitations in the existing datasets, I investigated myoblast cell lines from 10 different mammalian species under identical cell culture conditions representing no imposed stress (basal) and the stress of H_2O_2 exposure. The levels of two molecular chaperone proteins (HSP60 and GRP78) were measured by Western blot under both basal and imposed stress conditions.

I tested the hypothesis that cultured myoblasts from the longer-lived species included in this study would have higher basal levels of both molecular chaperones as well as a stronger induction of both molecular chaperons following exposure to H_2O_2.

Chapter 2: MLSP of endothermic vertebrates correlates with HSP60, but not with antioxidant enzymes GPx and GR in brain mitochondria

Background and Objectives:

Mitochondria have been implicated in many neurodegenerative diseases of aging due to their role in generating ROS, being a proximal target of ROS and participating in protein misfolding diseases. There are two broad mechanisms by which a long-lived species might combat these problems. The first involves the removal of ROS, and the second involves repairing damages to macromolecules. The primary objective of this chapter was to determine if isolated mitochondria from naturally long-lived mammalian and avian species have higher levels/activities of GPx and GR (involved in the removal of ROS) as well as higher levels of mitochondrial HSP60 (involved in protein repair) than naturally short-lived mammalian and avian species.

Hypothesis:

I hypothesize that isolated mitochondria from the naturally long-lived species included in this study will have higher activities of GPx and GR, as well as higher levels of HSP60.

Contributions:

I performed all aspects of the work described, including mitochondrial isolations, enzyme measurements, protein level measurements, statistical analyses, and chapter write-up.

2.1 Abstract

Mitochondria are critical organelles that yield the majority of cellular energy in the form of ATP through the process of oxidative phosphorylation. This process however is not perfect. As electrons travel along the electron transport chain, some will leak from the respiratory complexes and react with oxygen, producing the superoxide anion which can be further metabolized into the highly toxic H_2O_2 via manganese superoxide dismutase. H_2O_2 can react with iron which results in the formation of the highly toxic hydroxyl radical, which can cause damage to proteins. To determine whether longer-lived mammalian and avian species have higher activities of the enzymes involved in the metabolism of H_2O_2, namely GPx and GR, mitochondria were isolated from brain tissue for 7 mammalian and 2 avian species, with MLSP that vary between 3.5 years and 31 years. Generally, larger species live longer than smaller species, so two species who display unusually long lifespans for their body size, the naked-mole rat and big brown bat, were included in this study to control for the trend between body mass and lifespan. It was determined that the activities of GPx and GR do not correlate to species MLSP, both before and after accounting for mitochondrial abundance. Therefore, there appears to be no correlation between the GSH antioxidant defence system in isolated mitochondria and species MLSP. In addition to antioxidant capacity, I also wanted to determine whether the longer-lived species had a higher level of the major mitochondrial repair protein, HSP60, which could counter the effects of no difference in oxidant removal. HSP60 levels in isolated brain mitochondria were measured via Western blot, and it was determined that HSP60 levels in longer-lived species did display higher levels of HSP60.

2.2 Introduction

In 1972, Harman extended his Free Radical Theory of Aging (Harman, 1956), which posits that organisms age due to accumulated damage to cellular macromolecules as a result of free radicals, to include mitochondria as the source of free radical production (Harman, 1972). Since the introduction of this theory, there has been a substantial amount of research on mitochondria and their proposed role in aging (see Robb et al., 2009 for a detailed review). Perhaps the most well-known putative role of mitochondria in aging relates to their ability to produce the superoxide anion during oxidative phosphorylation (reviewed in Murphy, 2009), which can dismutate into other forms of toxic ROS and cause damage to cellular macromolecules (Harman, 1981). All tissues are susceptible to oxidative insults, however the brain has been said to be especially sensitive due to its high oxygen consumption. While the size of the brain may only contribute a small percentage to total body mass, it accounts for 20% of basal oxygen consumption in humans due to the high ATP demand of neurons, and this high oxygen consumption may result in higher superoxide production, and possibly higher levels of oxidative damage (Halliwell, 2001). Furthermore, many of the most well-known neurodegenerative diseases of aging occur in the brain, and are thought to result from dysfunctional mitochondria (Lin et al., 2006). For example, Parkinson's disease (a neurodegenerative disease that results in decreased motor ability and unregulated motor movement) has be induced in mice that were administered MPTP, which inhibits complex I of the electron transport chain (Heikkila et al., 1984). Similarly, rats that were administered injections of rotenone (another complex I inhibitor) also showed signs of Parkinson's disease (Cannon et al., 2009), suggesting that dysfunctional complex I may be linked to the disease.

Complex I inhibition could result in increased levels of superoxide (it is one of the sites of superoxide formation in the electron transport chain) which could cause further damage to the mitochondrial respiratory complexes, as well as to mitochondrial proteins and DNA, other cytosolic proteins, and nuclear DNA. Indeed, there is evidence for increased nuclear oxidative DNA damage (measured by the oxidative insult, 8-hydroxy-2$^{'}$-deoxyguanosine) (Alam et al., 1997; Zhang et al., 1999), and increased protein oxidative damage (measured by carbonyl levels) in patients with Parkinson's disease (Alam et al., 1997). Two of the most well studied proteins that are often abnormal in patients with Parkinson's disease include Parkin (codes for an ubiquitin ligase and involved in protein homeostasis), and Pink-1 (codes for a kinase involved in signalling), both of which are associated with mitochondria. Parkin is able to associate with the outer mitochondrial membrane to prevent cytochrome c release (Darios et al., 2003), and is also involved in mitochondrial biogenesis (Kuroda et al., 2006). Furthermore, Parkin-null *Drosophila* (Pesah et al., 2004) and mice (Palacino et al., 2004) show mitochondrial impairment, increased oxidative stress and symptoms related to Parkinson's disease. Similarly, Pink-1 is thought to protect against cell death (Petit et al., 2005), and Pink-1 null *Drosophila* are more sensitive to oxidative stress induced by paraquat and rotenone (Yang et al., 2006). Other neurodegenerative diseases that have demonstrated an association to dysfunctional mitochondria include Alzheimer's disease (Reddy et al., 2004; Li et al., 2002), Amyotrophic lateral sclerosis (Lin et al., 2006), Huntington's disease (Gu et al., 1996; Milakovic et al., 2005), and Friedreich's ataxia (Kaplan, 2002). Therefore, there is substantial evidence that implicate mitochondria in neurodegenerative diseases of aging.

The relationship between mitochondria and aging, combined with the limited replacement of damaged neurons over the course of an adult lifetime (Bhardwaj et al., 2006),

makes brain mitochondria particularly interesting to aging research. Dysfunctional mitochondria can induce apoptosis in neurons, which would result in a smaller population of functional cells over time. Eventually, the population of functional neurons would not be enough to maintain tissue function, and the brain will shut down resulting in the death of the organisms. Therefore, species that experience a higher MLSP might be able to maintain proper functioning neurons for longer, and thus, delay the onset of neurodegeneration.

Substantial progress has been made in identifying potential traits that allow long-lived species to maintain proper cellular functioning for a longer period of time. One of the most robust traits associated with longevity that has been discovered thus far is stress resistance. Many researchers have revealed a positive correlation between stress resistance and longevity both *in vivo* (Service et al., 1984; Rose et al., 1992; Lin et al, 1998; Larsen, 1993; Vanfletern, 1993; Lithgow et al., 1995; Kenyon et al., 1993; Bartke et al., 2001; Bokov et al., 2009; Flurkey et al., 2001; Panici et al., 2010; Murakami et al., 2003; Salmon et al., 2005) and *in vitro* (Kapahi et al., 1999; Harper et al., 2007; 2011; Csiszar et al., 2011), suggesting that there is a relationship between stress resistance and longevity, however the mechanisms involved in stress resistance are less understood. Two ways in which cells can negate damages caused by stress include the removal of damaging molecules and the repair of damaged macromolecules.

In mitochondria, there are numerous antioxidant enzymes that convert ROS to a less toxic form. MnSOD is perhaps one of the most important of these enzymes. The criticality of MnSOD was established by the observation that homozygous knockout mice die shortly after birth (Li et al., 1995), and show signs of aging, including neurodegeneration, myocardial injury, and lipid peroxidation (Lebovitz, 1996). Furthermore, MnSOD has been shown to correlate positively with species MLSP in brain tissue (Page et al., 2010). Additionally, MnSOD has been

shown to be involved in other cellular processes, including cell growth and proliferation, and mitochondrial fusion/fission, both of which have been implicated in aging (see Robb et al., 2014 for a detailed review). Catalase, which removes cytosolic H_2O_2 is also positively correlated with species MLSP in brain; however two other antioxidants involved in H_2O_2 removal, GPx and GR, do not correlate with species MLSP in whole brain, liver, or kidney tissue (Page et al., 2010). The GSH is important in maintaining cellular redox homeostasis and was initially thought to be important in establishing lifespan. Overexpression of multiple isoforms of GPx have resulted in increased stress resistance in mice (Dabkowski et al., 2008; Lie and Cheng, 2005; Ran et al., 2006) and lifespan in *D. melanogaster* (Mockette et al., 1999), suggesting that differences in GPx activity (and GR activity, which is required to maintain a pool of GSSG) might explain, at least in part, the vast disparity in lifespan across the mammalian kingdom. However, since there was no observable correlation between total cellular GPx/GR activities at the tissue level and species MLSP (Page et al., 2010), it remains to be determined whether the activities of the mitochondrial isoforms of GPx and mitochondrial fraction of GR correlate with species MLSP.

Removal of oxidants is a way in which mitochondria can prevent damage to macromolecules, however this strategy is not 100% effective. In order to compensate for damage to macromolecules, mitochondria possess other proteins that aid in repairing the damage. Proteins are particularly susceptible to oxidative insults due to the presence of thiol groups in some amino acids that are easily oxidized to disulfide bonds. This type of oxidative insult is particularly damaging because most amino acids which contain thiols are often located at the catalytic site of the protein, thus rendering the protein inactive (Thomas et al., 2001). Furthermore, if the oxidative insults are not removed, then the proteins can form aggregates, which are a characteristic of Parkinson's disease and Alzheimer's disease (Baba et al., 1998; Lin

et al., 2006). Therefore, cells must contain mechanisms which repair damaged proteins, thus maintaining their function and preventing aggregation. HSPs, also known as molecular chaperones, aid in the folding of newly synthesized proteins, as well as in the refolding of damaged proteins due to stress, and the prevention of protein aggregation (Calderwood et al., 2009). HSP60 is a mitochondrial matrix protein, however, there is evidence for the presence of HSP60 in the cytosol as well (Gupta et al., 2002). HSP60 is constitutively expressed and further induced by stress, and is required for the re-folding of proteins as they enter mitochondria, as well as re-folding any damaged proteins (Stetler et al., 2010). Furthermore, HSP60 expression is upregulated in response to stress (Haynes et al., 2007), and it has also been shown to be involved in the prevention of Alzheimer's disease in human neuroblastoma cells by removing amyloid-β peptide before aggregate formation (Veereshwarayya et al., 2006). Together, these data suggest that HSP60 may play a pivotal role in maintaining protein homeostasis in mitochondria, and as such, may contribute to establishing MLSP. Indeed, a positive correlation between HSP60 and species MLSP was observed in whole brain, heart, and liver tissues (Salway et al., 2011a), however due the presence of HSP60 in the cytosol, it remains to be determined whether HSP60 in mitochondria alone correlates with species MLSP.

In this study, I test the hypothesis that longer-lived species have higher activities of GPx and GR, as well as higher levels of HSP60, in isolated brain mitochondria. To address this, mitochondria were isolated from brain tissue of individuals representing seven mammalian species and two avian species. The inter-species comparison approach used in this study allows for the determination of which molecular traits have co-evolved with longevity in animals. This type of study has been used in the past on the relationship between MLSP and ROS production (Lambert et al., 2007), cellular stress resistance (Kapahi et al., 1999), and antioxidant enzymes

(Page et al., 2010), however there are limitations to the comparative approach which need to be addressed. First, body mass is highly correlated with species MLSP, and as such, any traits that correlate with body mass will also correlate with species MSLP. I have statistically controlled for the effects of body mass by calculating the residuals from the regression line (vertical distance from the line of best fit) of body mass and the trait of interest, as well as the body mass and MLSP (reviewed in Speakman, 2005). Secondly, when performing a comparative study, all species must be approximately the same age, which, for the purpose of this study, is young adulthood (sexually mature yet shows no signs of aging). This ensures that any traits found to correlate with MLSP are not a consequence of aging.

2.3 Materials and Methods

2.3.1 Materials

All chemicals were purchased from Bioshop (Burlington, ON, Canada), Fisher Scientific (Mississauga, ON, Canada) or Sigma Aldrich (Oakville, ON, Canada), unless otherwise stated. BioRad protein dye was purchased from BioRad Laboratories (Hercules, California, US). Prestained broad range protein marker was obtained from BioLabs, (New England, Massachusetts, USA). Pierce Memcode Reversible Protein Stain Kit™ was purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). Anti-HSP60 primary antibody (ab31115) was purchased from Abcam (Burlington, Ontario, Canada). Unconjugated IgG anti-rabbit (goat) secondary antibody was purchased from Rockland Immunochemicals (611-1102) (Gilbertsville, Pennsylvania, US).

2.3.2 Animals

Animal species were chosen so that as wide as possible an array of MLSP could be represented. Between 3 and 6 individuals per species (with a total of 9 mammalian and 2 avian species) were sampled (Table 2.1). All species were endothermic vertebrates with a similar basic body plan and physiology. Rats were obtained from Dr. Cheryl McCormick (Brock University) and euthanized at Brock University with injection of 1 mL of Euthanyl® (3 mL Euthanyl to 2 mL saline). Naked mole rats were obtained from Dr. Melissa Holmes (University of Toronto at Mississauga) and euthanized with 0.5 mL of Euthanyl®. Big brown bats were collected from Dr. Paul Faure (McMaster University, Hamilton, ON) and euthanized with 0.3 mL of Euthanyl®. Hamsters, gerbils, and Guinea pigs were obtained from Charles River, MA, and euthanized at

Brock University with 0.5 mL (hamsters and gerbils) or 1 mL (Guinea pigs) Euthanyl®. Quails were obtained from CroQuail Farms (St. Anns, Ontario, Canada) after being euthanized on site by cervical dislocation (performed by farm staff as per their protocol) and transported on ice to Brock University (about 30 min) for tissue harvesting. Geese were obtained from a local farmer after being euthanized by decapitation. Heads were transported on ice to Brock University (about 30 min) for further processing. Following euthanasia of all animals, tissues were (heart, liver, kidney) extracted and flash frozen in liquid nitrogen for future use. Whole brains were removed and used directly for mitochondrial isolation.

2.3.3. Isolation of mitochondria

Brain tissue was homogenized with 5 mL of 1xMSHE (210 mM mannitol, 70 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM ethylene glycol tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM dithiothreitol (DTT), balanced to pH 7.4) with 0.5% bovine serum albium (BSA) by 12 passes of a glass on glass Dounce homogenizer. Samples were spun at 1000 g for 5 minutes (sorvall® RC-5C plus). The pellet was resuspended in 4 mL of 1xMSHE with 0.5% BSA and spun again at 1000g for 5 minutes. Supernatants were pooled and spun at 10 000 g for 10 minutes. Pellets were resuspended in 500 uL of 1xMSHE with 0.5% BSA and layered onto a discontinuous Percoll gradient (3 mL of 23% on the bottom, 3 mL of 15% on the top) and spun at 10 000 g for 25 minutes. Mitochondria (beige band) were removed, resuspended in 8 mL of 1xMSHE without BSA, and centrifuged at 10 000 g for 10 minutes. The pellet was then resuspended in 300 uL 1xMSHE without BSA and stored at -80^0C for future use.

Table 2.1: Body mass and MLSP of the species included in this study. Species body mass and MLSP data are from AnAge (de Magalhaes et al., 2005).

Species (common name)	Species (scientific name)	Number of Individuals	MLSP	Body Mass (g)
Syrian hamster	*Mesocricetus auratus*	4	3.9	105
Norway rat	*Rattus norvegicus*	5	4.5	300
Japanese quail	*Coturnix japonica*	6	6.0	115
Mongolian gerbil	*Meriones unguiculatus*	6	6.3	53
Rabbit	*Oryctolagus cuniculus*	6	9.0	1800
Guinea pig	*Cavia porcellus*	4	12.0	728
Big brown bat	*Eptesicus fuscus*	5	19.0	23
Goose	*Anser anser*	3	26.0	3309
Naked mole rat	*Heterocephalus glaber*	4	31.0	35

2.3.4 Bradford Assay

The protein concentration of mitochondrial pellets was determined using a Cary 100 Bio-UV-visible spectrophotometer and a BioRad protein assay kit. A stock solution of BioRad reagent was made by 4 parts milli-q water and 1 part dye. 1 mL of stock solution was added to each cuvette and bovine serum albium (BSA) was used to make the standard curve with

concentrations of 0 ug/mL, 2 ug/mL, 5 ug/mL, and 10 ug/mL. 1 uL of each sample was then added to each cuvette and measured at 595 nm.

2.3.5 CS Activity

CS activity was measured at 412 nm using a Cary 100 Bio-UV-visible spectrophotometer with 1 mL cuvettes. Each cuvette contained 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.1 mM acetyl-coenzyme A (AcoA), 0.005% triton-X, all in 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), and 2 ug of crude mitochondrial protein. Samples were warmed to physiological body temperature (37°C for mammals, except naked mole rats (32°C) and 42°C for birds) and the assay was run for 2 minutes to obtain a background rate. 0.5 mM oxaloacetate was then added to initiate the CS reaction, and data was collected for an additional 10 minutes. The initial slope (background) was subtracted from the final slope to obtain the slope due to CS activity. Specific activity was then calculated using the equation: slope/extinction coefficient (13.6/mMcm)*(optical path length)/protein amount.

2.3.6 Lactate Dehydrogenase Activity

As a marker of cytosolic contamination, lactate dehydrogenase activity was measured in all mitochondrial fractions. The lactate dehydrogenase activity assay was performed spectrophotometrically as above, but at 340 nm. Each 1 mL cuvette contained 0.2 mM nicotinamide adenine dinucleotide (NADH), 2 ug crude mitochondrial protein, and 20 mM HEPES (pH 7.3). Cuvettes were warmed to physiological body temperature (as above), and a background rate quantified over 2 minutes. Following this, 10 mM pyruvate was added to initiate the lactate dehydrogenase reaction, and data was collected for an additional 10 minutes. The

initial slope (background) was subtracted from the final slope to obtain the slope due to lactate dehydrogenase activity. Specific activity was then calculated using the equation: slope/extinction coefficient (6.226/mMcm)*(optical path length)/protein amount.

2.3.7 GPx Assay

GPx activity was measured essentially as above at 340 nm, using 1 mL cuvettes. Each cuvette contained 50 mM potassium phosphate buffer (KPB) (pH 7.0), 1 mM GSSG, 0.15 mM nicotinamide adenine dinucleotide phosphate (NADPH), 1 U/mL GR, and 6 ug of crude mitochondrial protein. The cuvettes were warmed to physiological body temperature, and the background rate of absorbance change measured for 2 min. Then 0.0007% H_2O_2 was added to initiate the reaction and data was collected for an additional 10 minutes. The background was subtracted from the final rate to obtain the slope due to GPx activity. Specific activity was calculated using the same equation as for lactate dehydrogenase activity.

2.3.8 GR Assay

GR activity was determined as above at 340 nm, using 1 mL cuvettes which contained 75 mM KPB with 2.6 mM EDTA (pH 7.6), 0.09 mM NADPH, and 6 ug of crude mitochondrial protein. 10 mM GSSG was added to initiate the reaction. The initial slope (background) was subtracted from the final slope to obtain the slope due to GPx activity. Specific activity was calculated using the following equation: slope/extinction coefficient (6.226/mMcm)*(optical path length)/protein amount.

2.3.9 HSP60 Quantification

Crude mitochondrial protein (20 ug) was separated by SDS-PAGE on a 4% stacking, 12% resolving gel and electrotransferred to a polyvinylidene fluoride (PVDF) membrane. Beta actin (which was used in Chapter 3 as a loading control) is not present in mitochondria, and the widely used mitochondrial protein loading controls (COX IV and VDAC) are not 100% homologous for the species used in this study. Therefore, all membranes were stained with Memcode reversible protein stain prior to blocking and immunoblotting. This staining method allowed for determination of equal loading and even transfer. Any unevenly transferred membranes were discarded. After staining, membranes were blocked overnight (4°C) in a blocking solution that contained 5% skim milk in 1x phosphate buffer saline (PBS). Membranes were then probed for mitochondrial HSP60 (which is highly conserved across all species included in this study; Figure 2.1) using anti-HSP60 (1:200 dilution) primary antibody (specific to mitochondria) in 5% skim milk dissolved in 1x PBS Tween for 1 hour at room temperature or overnight at 4°C. Membranes were then rinsed 5 times at 5 minute intervals with 1x PBS Tween (1xPBS-t) (polyethylene glycol sorbitan monolaurate) (1ml Tween/1 L PBS) and probed with anti-rabbit secondary antibody for 1-2 hours at room temperature. Membranes were washed again with 1x PBS-t 4 times at 5 minute intervals, and once with 1x PBS for 5 minutes. Protein quantification was performed using the Odyssey infrared imaging system (Version 1.0; LI-COR Biosciences). For each membrane, band intensities were standardized to a rat sample that served as an internal control, and further standardized to CS activity to provide a per mitochondria value. Each measurement was made in duplicate, with the location of each individual being randomly varied within the two gels.

2.2.10 Statistical Analysis

All raw data (trait of interest, MLSP, and body mass) were natural-logarithm (Ln) transformed prior to correlation analyses since the relationships between MLSP or body mass and the variables of interest appeared to be power functions which can be estimated using log-transformation on both axes (Page et al., 2012). Residuals for both the trait of interest as well as MLSP were calculated from simple linear regressions with body mass as the independent variable, and the trait of interest or MLSP as the dependent variable. These residuals were plotted together with the residual of MLSP as the dependent variable, and the residual of the trait of interest as the independent variable, to remove the confounding effect of body mass (Speakman, 2005). A Pearson Product Moment correlation analysis was used to determine significance.

Rat	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Hamster	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Rabbit	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Guinea Pig	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Gerbil	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Naked Mole Rat	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Big Brown Bat	LTRAYAKDVKFGAEARALMLQGVDLLADAVAVTMGPKGR
Chicken	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Turkey	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Zebra Finch	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR

Figure 2.1: Sequence alignment for HSP60 in mammals and birds. Partial sequence alignment for HSP60 showing the region in which amino acid sequence identity is highly conserved and the epitope (in red) to which the antibody was raised. Sequences for quail and goose were not found in protein databases; however due to the 100% homology between other bird species and mammalian species, it is assumed that sequences for both quail and goose are also 100% conserved.

2.4 Results

2.4.1 MLSP is not correlated to mitochondrial GPx activity in isolated brain mitochondria from endothermic vertebrates.

To determine if the relationship between stress resistance and longevity seen in endothermic vertebrates was associated with increased mitochondrial antioxidant capacity the activity of GPx was measured in isolated brain mitochondria of 7 mammalian and 2 avian species. Since physiological body temperature varied over 10°C in the species used in this study, and all enzyme activities were measured at physiological body temperature, antioxidant enzyme activities were standardized to CS as an index of mitochondrial activity (Larsen et al., 2012). Note that there was no correlation between CS activity and species MLSP (Appendix I, Figure 1). GPx activities were calculated, standardized to species aerobic capacity (CS activity) (Table 2.2), and then natural logarithm (Ln) transformed. A correlation analysis revealed no significant correlation of MLSP with GPx activity (Figure 2.1A). The analysis was repeated using data not standardized to CS and a similar result obtained (Figure 2.2A). To test whether a relationship was confounded by possible body mass effects, residuals of body mass vs. MLSP (Appendix I, Figure 3) were plotted against the residuals of body mass vs. GPx activity (both standardized and not standardized to CS) (Figure 2.1B and 2.2B). This analysis also revealed no correlation between MLSP and GPx. Lactate dehydrogenase activities were measured for all species included which confirmed that there was no cytosolic contamination (data not shown).

Table 2.2. Non-transformed data for GPx activity (not standardized and standardized to aerobic capacity (GPx/CS)) in isolated brain mitochondria from 9 endothermic vertebrate species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM).

Species	MLSP	GPx (µmol/min/mg)	SEM	GPx/CS (x1000)
Norway rat	4.5	3.75	0.298	0.0118
Japanese quail	6.0	7.88	0.254	0.0526
Mongolian gerbil	6.3	1.95	0.354	0.0223
Rabbit	9.0	2.01	0.263	0.0142
Guinea pig (GP)	12.0	4.56	0.917	0.0382
Big brown bat	19.0	2.30	0.253	0.0526
Goose	26.0	11.6	1.23	0.114
Naked mole rat (NMR)	31.0	0.720	0.230	0.00920

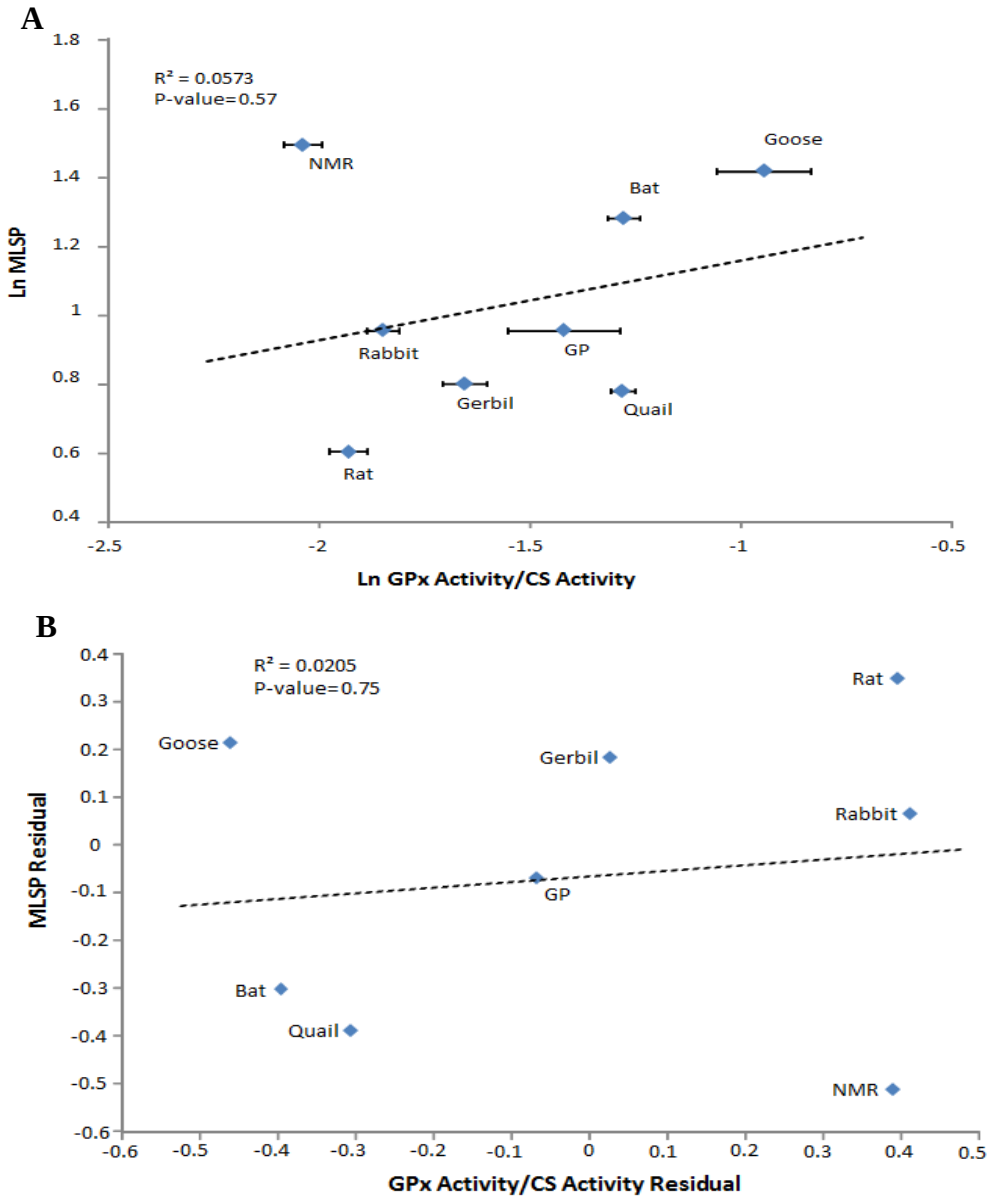

Figure 2.2: MSLP as a function of GPx activity in isolated brain mitochondria from endothermic vertebrates after being standardized to species aerobic capacity. A) MSLP is not correlated to GPx activity. B) Residual analysis of MSLP is not correlated to GPx activity. GPx activity was measured and standardized to the species aerobic capacity (measured as CS activity (µmol/min/mg) for nine endothermic vertebrate species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual).

2.4.2. MLSP is not correlated to mitochondrial GR activities in isolated brain mitochondria from endothermic vertebrates.

GR activity was measured in isolated brain mitochondria from representatives of 7 mammalian and 2 avian species. As with GPx, GR activities were calculated, standardized to CS activity (Table 2.3), and then natural logarithm (Ln) transformed. A correlation analysis revealed no significant correlation of MLSP with GR activity. The same result was obtained when GR data were not standardized to CS (Figure 2.3A and 2.4 A). To test whether a relationship was obscured by body mass effects, residuals of body mass vs. MLSP were plotted against the residuals of body mass vs. GR activities (standardized and not standardized to aerobic capacity) (Figure 2.3B and 2.4B). This analysis also revealed no correlation between GR activity and MLSP.

Due to the observation of high GPx activity and low GR in both avian species, a ratio of GPx activity to GR activity was calculated for all species to see if perhaps, this ratio correlated to species MLSP. It was determined that there is no relationship between this ratio and species MLSP (Appendix I, Figure 2).

Table 2.3. Non-transformed data for GR activity (not standardized and standardized to aerobic capacity (GR/CS)) in isolated brain mitochondria from 9 endothermic vertebrate species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM).

Species	MLSP	GR (µmol/min/mg)	SEM	GR/CS
Hamster	3.9	1.39	0.252	0.0101
Norway rat	4.5	3.47	0.591	0.0109
Japanese quail	6.0	1.24	0.164	0.0120
Mongolian gerbil	6.3	3.40	0.494	0.0397
Rabbit	9.0	1.42	0.380	0.0253
Guinea pig (GP)	12.0	2.37	0.373	0.0178
Big brown bat	19.0	2.55	0.170	0.0629
Goose	26.0	1.22	0.282	0.00820
Naked mole rat (NMR)	31.0	1.53	0.152	0.0201

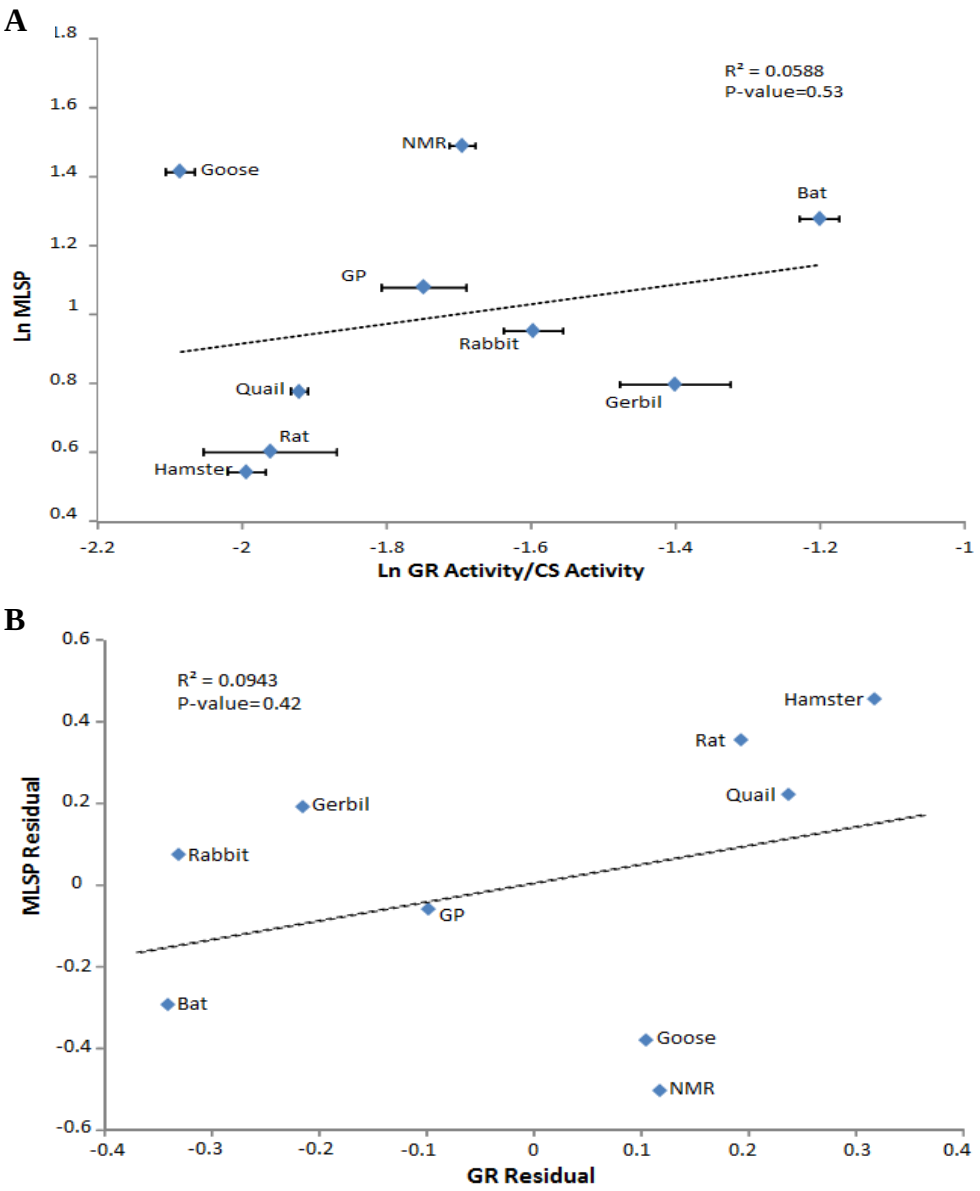

Figure 2.3: MSLP as a function of GR activity in isolated brain mitochondria from endothermic vertebrates after standardization to species aerobic capacity. A) MLSP is not correlated to GR activity. B) Residual analysis of MLSP is not correlated to GR activity. GR activity was measured and standardized to the species aerobic capacity (measured as CS activity (µmol/min/mg)) for nine endothermic vertebrate species, with 2-6 individuals per species. All measurements were made in duplicate.

2.4.3. MLSP is correlated to HSP60 levels in isolated brain mitochondria from endothermic vertebrates.

Mitochondrial HSP60 was measured via Western blot in isolated brain mitochondria of 7 mammalian and 2 avian species. HSP60 levels were determined, standardized to an internal control (Rat) and the species aerobic capacity (CS activity) (Table 2.4), and then natural logarithm transformed (Ln). A correlation analysis revealed a significant positive correlation of MLSP with HSP60 levels, but only after being standardized to the species aerobic capacity. To test whether the lack of a relationship between MLSP and raw HSP60 levels was hidden due to body mass, residuals of body mass vs. MLSP were plotted against the residuals of body mass vs. HSP60 levels (raw data) (Figure 2.6B) This analysis did not reveal any correlation. The same analysis was performed on the standardized HSP60 levels to determine whether the observed positive correlation was driven by body mass, however this analysis did not change the correlation between HSP60 levels and MLSP suggesting that HSP60 levels in isolated brain mitochondria from endothermic vertebrates are correlated to species MLSP, but only after accounting for the species aerobic capacity.

Table 2.4. Non-transformed data for HSP60 levels (not standardized and standardized to aerobic capacity (HSP60/CS)) in isolated brain mitochondria from 9 endothermic vertebrate species. Each value is the average for 2-6 individuals of each species (run in duplicate). Intensities for HSP60 from each individual were compared to an internal standard control (rat) which simultaneously resolved within every gel. The standard error of the mean is also shown (SEM).

Species	MLSP	HSP60 Intensity (Standardized to Rat)	SEM	HSP60/CS
Hamster	3.9	1.49	0.130	0.100
Norway rat	4.5	1.32	0.030	0.410
Japanese quail	6.0	1.28	0.16	1.27
Mongolian gerbil	6.3	1.23	0.29	0.700
Rabbit	9.0	0.970	0.080	1.64
Guinea pig (GP)	12.0	1.34	0.26	1.08
Big brown bat	19.0	1.23	0.16	3.07
Goose	26.0	1.53	0.11	1.01
Naked mole rat (NMR)	31.0	2.36	0.30	2.96

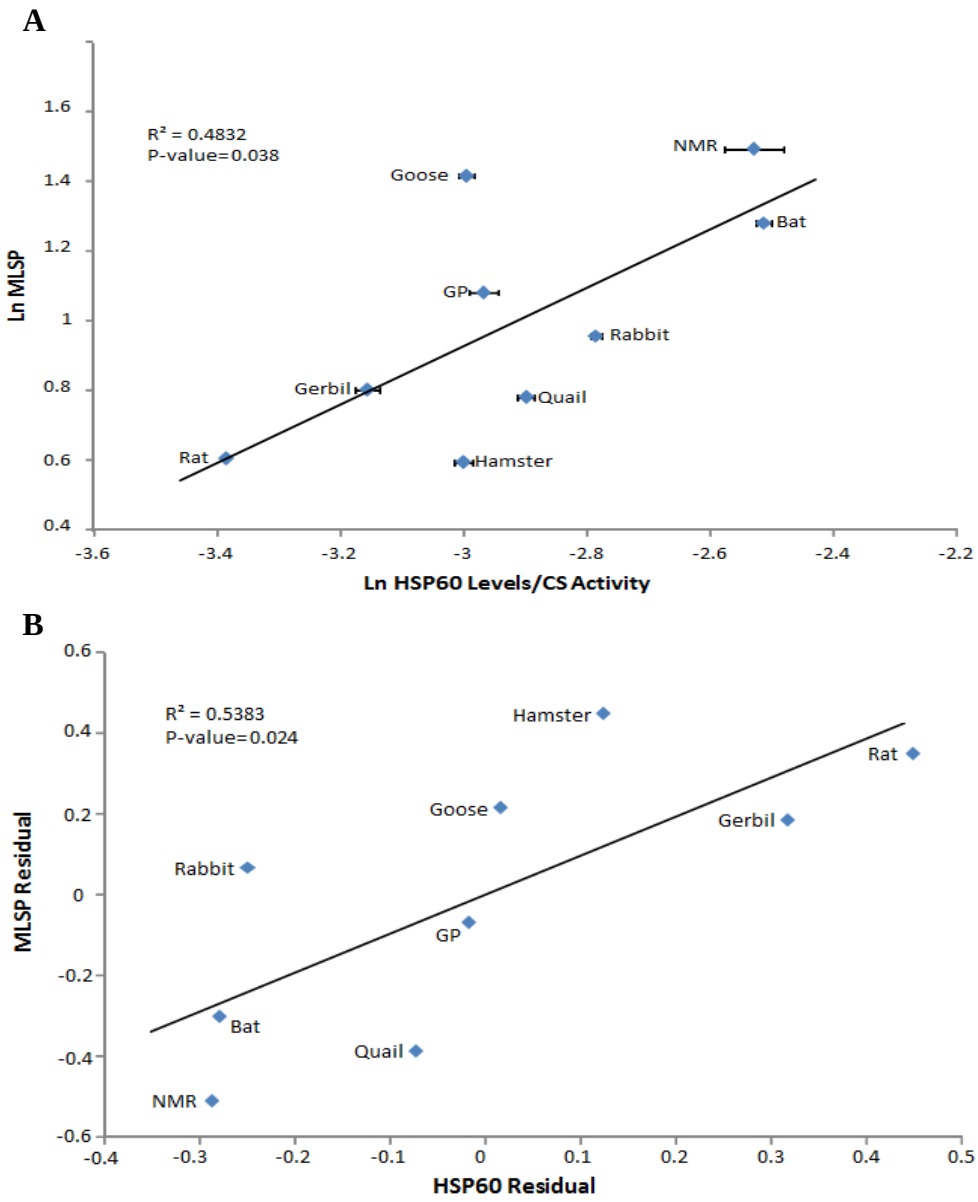

Figure 2.4: MLSP as a function of HSP60 levels in isolated brain mitochondria from endothermic vertebrates after standardization to species aerobic capacity. A) MLSP is positively correlated to HSP60 levels. B) Residual analysis of MLSP is positively correlated to HSP60 levels. HSP60 levels were measured and standardized to the species aerobic capacity (measured as CS activity (µmol/min/mg) for nine endothermic vertebrate species, with 2-6 individuals per species. All measurements were made in duplicate and standardized to an internal control (rat).

2.5 Discussion

Results from studies that manipulate levels of protein via genetic modifications are inconsistent and fail to provide compelling case for a role of antioxidant enzymes in conferring longevity (reviewed in Perez et al., 2009b). The comparative approach used here provides an alternative method to study the role of certain proteins without facing many of the challenges presented by genetic manipulations (e.g. possible secondary effects that could account for any observable changes, effects related to global life-long over- or under-expression). However, there are a couple limitations from this thesis that need to be addressed. First, the two bird species included in this study represent approximately 20% of the total sample size. Since bird speceis are generally long lived for their body size, the inclusion of two bird species in this study (which included 9 species altogether) could result in skewed data. However, the Japanese quail is the shortest lived bird species (according to AnAge), and has a relatively close MLSP to the size matched rat species. This allows for a fair comparison to be made between the Japanese quail and the goose which is a larger, longer-lived bird species. Secondly, the naked mole rat and big brown bat are also exceptionally long lived for their body size and also account for 20% of the total sample size and could also result in skewed data. Nonetheless the results from the present study are consistent with those from previous studies suggesting that the uniqueness of the species included in this study does not affect the results.

This study utilized a broad species comparative approach to measure mitochondrial GPx and GR activities from species of disparate natural longevities, and found no correlation between species MLSP and GPx or GR activities. A separate comparative study which analyzed GPx and GR activity at the whole tissue level, in brain, liver, and heart of 14 species (including mammals and birds; MLSP ranging from 3.5 years to 122 years) came to the same conclusion. Similarly no

correlation of GPx or GR with MLSP was observed in whole brain tissue (Page et al., 2010). Previously, some researchers reported a positive correlation between species MLSP and GPx activity in brain (Sohal et al., 1990a), but a negative correlation in heart and liver (Sohal et al., 1990a). While the results from these two studies contradict one another, it seems logical that there is no correlation between GPx activity and species MLSP. There is more statistical power in the study by Page et al., 2010, which found no correlation between GPx activity and MLSP, as they included more than double the number of species than the study done by Sohal et al., 1990a. In addition, the results from this thesis also indicate no correlation between MLSP and GPx activity.

Results from two species comparisons are also equivocal. In a two species comparison between the naked mole rat (MLSP 31 years) and a normal lab mouse (MLSP 3.5 years) it was determined that GPx activity in liver tissue in naked mole rat was approximately 70 times lower than in mice (Andziak et al., 2005). This is consistent with the results of this study, in which the naked mole rat had the lowest GPx activity amongst all species included, and it was approximately 80% lower than the rat, which has a similar MLSP of the mouse (MLSP of 4.0 years as opposed to 3.5 years for a mouse). However, other long-lived species showed higher levels of GPx activity when compared to a size matched species with a lower MLSP (Ku et al., 1993). GPx activity in pigeon heart and brain was significantly higher than in the rat, which is also consistent with the results from this study, as the Japanese quail, which is similarly sized to the rat, had approximately double the GPx activity. Due to the inconsistent results of GPx activity and MLSP, and the fact that the studies that did find a correlation were severely limited in the number of species used (and thus have weak statistical power), it can be suggested that there is no role for GPx in establishing species MLSP.

It is interesting to note that GSH (which is needed for GPx and GR activity) is present at lower levels in longer lived species in brain, heart (Sohal et al., 1990a), liver (Sohal et al., 1990a; Perez-Campo et al., 1994) and lung (Lopez-Torres et al., 1993). However, when comparing GSH levels in pigeon to rat, it was found that the pigeon (which is longer lived) had higher levels of GSH in kidney and brain, but equal levels in heart tissue (Ku et al., 1993). A limitation of many of these studies is the limited sample size used. Most of these studies only included between 2-6 species, resulting in low statistical power, and the species were not confined to a single class (mammals, birds, and amphibians were included in the studies that looked at GSH in liver, and lung), so the results obtained could be due to difference in body plans, the ability to fly, or due to being an endotherm vs. an ectotherm. In addition, studies with a low sample number can also be greatly affected by outlying data points, leading to biased results. Furthermore, the results from the two species comparison (which suggest GSH levels do correlate with MLSP) must also be taken with caution, as two species cannot provide enough statistical power to prove a correlation exists (Garland et al., 1994). Regardless of the limitations of the studies, it is possible that GSH levels are lower in long lived species as each study presented the same results. Therefore, it would be interesting to revaluate GPx and GR activity in both tissue homogenates and isolated mitochondria from the included species in this study, but standardized to GSH levels. It is possible that GPx and GR activity in the longer-lived species are actually higher than the short-lived species, but due a lower level of total GSH, this increased activity cannot be seen as GSH levels could be rate limiting.

Taken together, there appears to be no correlation between GPx or GR activity and species MLSP, however there are other antioxidant enzymes present which could account for the increased lifespan seen in some species. When levels and activities of CuZnSOD, catalase were

measured in liver, brain, and heart tissues from 6 different mammalian species (MLSP ranging from 3.5 years to 30 years) it was determined that species MLSP correlated positively with CuZnSOD activity in all three tissues, whereas catalase was positively correlated with species MLSP only in heart (Sohal et al., 1990a). However, in a separate comparative study which included 14 mammalian and avian species (MLSP ranging from 3.5 years to 122 years) CuZnSOD did not correlate with species MLSP in heart, liver, or brain tissue, and catalase did not correlate with species MLSP in heart, but was positively correlated in brain tissue (Page et al., 2010). Similarly, two other antioxidant enzymes, glutaredoxin and thioredoxin, failed to reveal any significant correlation with species MLSP in heart, liver, or brain tissue when measured in 15 mammalian and avian species (Salway et al., 2011b). Together, these data provide equivocal evidence for a role of antioxidant enzymes in determining MLSP.

One particular antioxidant enzyme that has been shown to be involved in determining longevity is MnSOD. MnSOD was positively correlated with species MLSP in brain tissue (Page et al., 2010), and is essential for survival (Li et al., 1995). MnSOD is particularly interesting as it has been shown to slow cellular proliferation and growth (Lin et al., 1998; Ough et al., 2004). The ability of MnSOD to protect the replicative capacity of cells *in vivo* could contribute to longevity, as a slowed rate of division could extend the replicative capacity of these cells, thus delaying the onset of senescence. In addition, MnSOD is also thought to promote fusion of mitochondria. Mitochondria can either be filamented (fused), which results in increased membrane potential, resistance to permeability transition, (Detmer and Chan, 2007), and resistance to stress induced cell death (Shutt and McBride, 2013), or fragmented. While the role of MnSOD in mitochondria fusion is novel, and therefore research is somewhat limited, it is thought to be involved as overexpression of MnSOD results in similar characteristics of

mitochondrial fusion, including increased membrane potential, reduced permeability transition (Silva et al., 2005), and stress resistance (Shan et al., 2007). However, due to the limited research on this role of MnSOD, it remains to be determined whether it is directly involved in mitochondrial fusion.

If stress resistance was indeed a mechanism that is involved in delaying the aging process, than one could expect to see a parallel trend in the production of the damaging oxidants and lifespan, i.e. longer-lived species would show lower levels of ROS generation. Indeed, many comparative studies have revealed a negative correlation between ROS generation (measured primarily as SO and H_2O_2) (Sohal et al., 1989; Sohal et al., 1990b; Ku et al., 1993; Barja, 2002; Lambert et al., 2007), however there are many problems with data collected that analyze ROS levels. Firstly, superoxide is not a suitable measurement of ROS levels. Probes used to detect superoxide must have access to the matrix where they will have to compete with MnSOD, which is present at high levels in the matrix (Fridovich, 1995). Secondly, other forms of ROS are also detoxified by antioxidant enzymes which could vary between experimental groups or species (in the comparative approach). Third, the oxygen concentrations in many of these studies on isolated mitochondria are at least an order of magnitude higher than mitochondria are exposed to *in vivo*. In addition, most of the superoxide formed under experimental conditions is though reverse electron transport, which has not been shown to occur *in vivo*. Taken together, this suggests that the actual levels of superoxide production *in vivo* are much less than what studies have typically found (reviewed in Murphy, 2009), and the observed trends with respect to MLSP should be interpreted with caution.

Together, there is not enough evidence from the literature to unequivocally conclude that mitochondrial ROS determines MLSP, and as such, the majority of antioxidant enzymes also

appear to have little to no role in determining MLSP (Robb et al., 2014; Sohal et al., 1990a; Page et al., 2010; Lambert et al., 2007). They only antioxidant enzyme that seems to have any role is MnSOD, but this role might not be so much as to detoxify superoxide to prevent damages, but rather to monitor superoxide levels to maintain proper signalling within the cell (to control cell growth and possibly fusion and fission). Therefore, the literature fails to present a compelling case for the free radical theory of aging; so perhaps, longevity is established by other cellular processes.

One process that is thought to be involved in establishing longevity is protein homeostasis. Recall that aged individuals exhibit increased protein oxidation and aggregation, both of which are staples of neurodegenerative diseases of aging (reviewed in Ali et al., 2010). The proteins involved in oxidative phosphorylation are highly susceptible to oxidative insults due to their close proximity to mitochondria generated ROS and damaged complexes can result in a higher level of superoxide production. Furthermore, proteins that are nuclear encoded and sent to mitochondria must first unfold as they enter the organelle, where they are refolded back to their native structures. Therefore, protein homeostasis is very important in mitochondria. HSP60 is a mitochondrial HSP which is responsible for the refolding of proteins as they enter mitochondria, as well as refolding damaged proteins. In this study, it was found to correlate positively with species MLSP, suggesting it could be important in conferring longevity. This is congruent with results from a wide spread comparative study, in which 13 mammalian and avian species were analyzed for HSP levels in whole brain, heart and liver tissue. In all three tissues, MLSP was positively correlated to HSP60 levels. In addition, the levels of the major cytosolic HSP, HSP70, and the major endoplasmic reticulum chaperones, GRP78 and GRP94, were also highly correlated with species MLSP in all three tissues (Salway et al., 2011a), suggesting that

protein homeostasis might be important in conferring lifespan. However, it should be noted that the levels of HSPs do not provide any information on the activity levels, which may or may not correlate with lifespan.

In addition to unfolding due to oxidative insults, proteins can also undergo oxidation of redox-sensitive thiols, resulting in disulfide bridge formation which interferes with normal protein function. Reversal of this oxidative damage is catalyzed by a number of enzymes using the reducing power of GSH and NADPH. Two such proteins, glutaredoxin and thioredoxin, were studied in brain, heart, and liver tissue of 15 species, where results revealed no correlation between the activities of either protein and species MLSP in any of the three tissues (Salway et al., 2011b). Furthermore, the activities of these proteins were marginally lower in Snell dwarf mice compared to normal mice (Salway et al., 2011b), and glutaredoxin activity in the naked mole rat was similar to the activity of the common mouse (Perez et al., 2009a). Similar results were seen in protein degradation machinery. The 20S and 26S proteasomes are required for the degradation of damaged proteins following oxidative stress (Divald et al., 2006), thereby preventing aggregation of damaged proteins. However, a comparative study which included both mammalian and avian species failed to reveal a correlation between MLSP and the activities of both the 20S and 26S proteasome (Salway et al., 2011b). Furthermore, 20S proteasome activity was actually found to be significantly lower in brain tissue from long lived Snell dwarf mice when compared to normal laboratory mice, however 26S proteasome activity was similar amongst both experimental groups (Salway et al., 2011b). Similar results were also observed in a two species comparison between two long lived bat species (*Tadarida brasiliensis* and *Myotis velifer*) and mice, wherein proteasome activity was lower in liver tissues from the bat species (Salmon et al., 2009). Together, these data suggest that protein repair via redox regulating

enzymes and degradation via the 20S and 26S proteasome is not involved in establishing MLSP in mammalian and avian species. Thus, HSPs appear to be the only proteins involved in protein homeostasis that correlate with species MLSP, however it should be noted that HSP expression is mediated through IGF-1 signalling. IGF-1 inhibits the transcription of HSPs, and the levels of IGF-1 are thought to be negatively correlated to species body mass. This relationship, combined with the positive relationship between body mass and MLSP (Stuart et al., 2010), suggests that smaller animals have increased levels of IGF-1, and thus will have lower levels of HSPs, which was found to be true for HSP60. This pathway of HSPs transcription could explain why HSPs are higher in the longer lived species, and as such, the increased HSP60 levels observed in the longer-lived species in this study could simply be a consequence of IGF-1 signalling, rather than being selected for during the evolution of longevity. Interestingly, there is recent evidence that suggests the naked-mole rat has lower IGF-1 signalling, as these animals have decreased expression of genes involved in IGF-1 signalling (Kim et al., 2011); however more evidence is needed to further support this finding.

In conclusion, this study strengthens the argument against the free radical theory of aging. There is little evidence for decreased ROS production in longer-lived species, and the data surrounding antioxidant enzymes are equivocal; the only antioxidant enzyme that appears to be involved in longevity is MnSOD, but its role in longevity might be more in the way of regulating ROS levels than actually removing it to prevent damages. Furthermore, there is limited evidence for protein repair enzymes in longevity, with the exception of HSPs, however the correlation between HSPs and MLSP could be driven by the higher levels of IGF-1 in shorter-lived species.

Chapter 3: Basal expression and induction of two molecular chaperones, HSP60 and GRP78, and their role in longevity in cultured mammalian myoblasts.

Background and Objectives:

Mammalian MLSP has been shown to correlate with stress resistance both *in vivo* (whole organism) and *in vitro* (using fibroblasts). Furthermore, overexpression of HSPs, (which are constitutively expressed and stressed induced and involved in protein homeostasis) leads to increased stress resistance and lifespan. Therefore, the primary objective of this project was to determine if cultured myoblasts from naturally long-lived mammalian species have higher levels of HSPs at the basal level, and a larger induction of the proteins following exposure to stress, than naturally short-lived mammalian and avian species.

Hypothesis:

I hypothesize that myoblasts from longer-lived species will have higher levels of HSP60 and GRP78 under basal conditions and will show a greater induction of HSP60 and GRP78 following exposure to H_2O_2.

Contributions:

I performed all aspects of the work described, including cell culture, cell lysates, enzyme measurements, protein level measurements, statistical analyses, and chapter write-up.

3.1 Abstract

It is strongly suggested from the literature that cellular stress resistance correlates with lifespan. This trend has been observed at the level of whole organisms and tissues, as well as at the cellular level; however, the majority of research at the cellular level is limited to fibroblasts. A variety of stressors have been administered to cultured fibroblasts from mutant mice and their wild-type littermates and it was determined that fibroblasts from long-lived mutant mice (Ames, Snell, and GHR-KO) are more resistant to paraquat, heavy metals, rotenone, H_2O_2 (H_2O_2), UV irradiation, and heat stress than their non-mutant littermates (Panici et al., 2010; Murakami et al., 2003; Salmon et al., 2005). Furthermore, cultured fibroblasts from naturally long-lived mammalian species are more resistant to a range of stressors including paraquat, heavy metals, and H_2O_2 (Kapahi et al., 1999; Harper et al., 2007; 2011; Csiszar et al., 2011). Together, these data suggest that there is a correlation between stress resistance and lifespan, yet this trend has only been observed in a single cell type. Therefore, to determine whether this trend is only observable in fibroblasts, I cultured mammalian myoblasts (precursor cells to muscle cells), administered H_2O_2 for various time points, and measured two molecular chaperones, HSP60 and GRP78. These two proteins were measured by Western blot both at the basal level, as well as after H_2O_2 exposure. At the basal level, HSP60 does correlate with species MLSP, but only after accounting for mitochondrial abundance. In contrast, GRP78 does not correlate with species MLSP in the analyses performed here. Additionally, the magnitude of HSP60 induction is significantly higher in myoblasts from the longer-lived species after 1 hour of H_2O_2 exposure. There is no correlation between HSP60 induction and species MLSP following 3 hour H_2O_2 exposure or a 3 hour recovery period. GRP78 induction is not correlated to species MLSP regardless of the duration of H_2O_2 exposure. Therefore, these results suggest that HSP60 might

be important in stress resistance in mammalian myoblasts, but GRP78 is not involved in determining species longevity.

3.2 Introduction

A hallmark of aging is the degeneration of tissues and organs, which often result in organ failure and death. This process has been particularly well characterized in skeletal muscle, which undergoes a progressive age-associated decline that can eventually limit physiological capacities and performance. This process is termed sarcopenia (Rosenberg, 1989), and is hypothesized to occur in aged individuals for two reasons. Firstly, muscle cells are thought to be particularly susceptible to oxidative insults due to their high demand for ATP (and subsequently more mitochondria), which could result in increased oxidative phosphorylation and ROS production (Fulle et al., 2004). This higher level of ROS can contribute to the demise of muscle tissue due to the accumulation of damages caused by ROS. There is evidence for increased oxidative damage to lipids and DNA in aged muscle (Mecocci et al., 1999), as well as damages to the protein complement of the cell. For example, the ryanodine receptors (which are responsible for calcium release from the sarcoplasmic reticulum, which is a critical step in muscle contraction) are major targets of oxidative damage due to their large number of free thiols which are easily oxidized (Belia et al., 1998; Eu et al., 2000). Secondly, aged muscle tissues experience a substantial decline in regenerative capacity. Muscle-specific stem cells or satellite cells, are quiescent, progenitor cells that are responsible for the growth, repair and maintenance of skeletal muscle. In response to injury, satellite cells are activated and differentiate into a proliferative myogenic precursor cell called a myoblast (Tedesco et al., 2010). There are two ways that between species differences in satellite cells and their progeny myoblasts might contribute to aging. First, a larger satellite cell pool would allow for more muscle regeneration capacity, thereby maintaining functional muscle tissue longer resulting in a later onset of muscle degeneration. However, to date there is no information on whether longer lived species have a larger pool of available

satellite cells than shorter lived species. Second, the more capable the satellite cells are of maintaining their replicative capacity over time, the longer they will be able to remain functional and replace damaged, lost myofibres. Therefore, it is plausible that longer-lived animal species have superior mechanisms for maintaining functional pools of satellite cells and myoblasts and that this relates to their ability to maintain muscle function over a longer period of time. Longevity is associated with increased resistance to exogenous stressors in other cell types, such as fibroblasts (Kapahi et al., 1999; Harper et al., 2007; 2011; Csiszar et al., 2011). It is possible that this property of stress resistance manifests also in myoblasts of longer-lived species.

There is little information available on stress resistance in muscle precursor cells; however there is substantial information on stress resistance in fibroblasts, which are precursor cells required for the maintenance of the extracellular matrix. A variety of stressors have been administered to cultured fibroblasts from long-lived mutant mice and their wild-type littermates and it was determined that fibroblasts from long-lived mutant mice are more resistant to stress than their non-mutant littermates (Panici et al., 2010; Murakami et al., 2003; Salmon et al., 2005). In addition, this relationship between stress resistance and longevity has also been observed in cultured fibroblasts from naturally long-lived mammalian species, where these cells were more resistant to various stressors than fibroblasts from shorter-lived mammalian species (Kapahi et al., 1999; Harper et al., 2007; 2011; Csiszar et al., 2011). Taken together, there appears to be a correlation between stress resistance and longevity in fibroblasts, however since this relationship has mainly been studied in a single cell type, it cannot be said true of other cell types.

The cellular and molecular mechanisms conferring stress resistance and longevity are also not completely understood. It is strongly suggested from the literature that protein

homeostasis plays an important role, as increased protein damage resulting in aggregate formation is a prominent characteristic of neurodegenerative diseases (Reviewed in Ali et al., 2010). The importance of protein homeostasis is further illustrated by the unfolded protein response (UPR) that is found in various cellular components (cytosol, mitochondria, and endoplasmic reticulum (ER)). This response becomes initiated when there is an accumulation of unfolded and/or damaged proteins in any of these compartments. Once initiated, this response results in increased levels of molecular chaperones which can aid in the refolding of the damaged proteins. The magnitude of the UPR decreases with age, which could explain why there is an observable increase in protein damage at older ages (Sherman M.Y., et al., 2001; Winklhofer K.F., et al., 2008). Similarly, the main effectors of the UPR, molecular chaperones, have also been shown to be involved in the aging process. Molecular chaperones, or HSPs, are constitutively expressed as well as induced by stressors (heat, oxidative stress, UV light) that activate heat shock factor 1 (HSF1) (transcription factor for HSP) (Murshid et al., 2013).

Data from genetic manipulations suggest a role for HSPs in aging, however the data are equivocal. Reducing the levels of HSP70 (a major HSP in the cytosol) in *C. elegans* resulted in advanced aging (Kimura et al., 2007), and overexpression of HSP-6 (Yokoyama et al., 2002), and HSP-16 (Walker and Lithgow, 2003) in these organisms resulted in increased lifespan. Similarly, overexpression of Hsp70 and HSF1 in disease models of mice (ALS and prion disease, respectively) also resulted in increased lifespan (Gifondorwa et al., 2007; Steele et al., 2008); however, overexpression of HSP70 in normal mice does not result in extended lifespan (Vanhooren et al., 2008). Furthermore, Snell dwarf mice have lower levels of HSP60 in heart tissue compared to normal littermates, but no significant differences between HSP70 and GRP78 levels were observed in this tissue (Salway et al., 2011a). Similar results were found in brain

tissue, wherein there were no significant differences in the levels of any of the three HSPs between Snell dwarf mice and normal littermates (Salway et al., 2011a). Together, these data cannot confirm or refute a role for HSPs in conferring longevity; however further evidence that support of a role for HSPs in longevity derives from studies that utilize the comparative approach.

In Chapter 2 it was observed that HSP60 levels in isolated brain mitochondria from longer-lived species were higher than in shorter lived species. In support of these findings, a separate comparative study (which included 15 mammalian and avian species) also found a positive correlation between lifespan and HSP60 levels, however this trend was observed in whole tissues rather than isolated mitochondria (Salway et al., 2011a). In addition, GRP78 and HSP70 are also elevated in all three tissues in the longer-lived species, suggesting that HSPs are associated with longevity. It is important to note, however, that these studies analyzed HSP levels at the basal level; no stress was imposed on the organism prior to sampling. Studies that have studied this feature of HSPs demonstrate their ability to be induced following stress (heavy metals, oxidants, radiation, and nitric oxide) (Landry et al., 1982; Li et al., 1982; Subjeck et al., 1982; Samli et al., 1998; Wagner et al., 1999). There is no information on whether the induction of HSPs is correlated to MLSP however. Due to the observations of increased stress resistance in fibroblasts from longer-lived species, higher HSPs in tissues from longer-lived species, and an ability of HSPs to be induced, it seems plausible that longer-lived species might have a more robust induction of HSPs following stress.

In this study, I test the hypothesis that cultured myoblasts from longer-lived species will have higher levels of HSP60 and GRP78 under basal conditions, as well as a more robust induction of these HSPs following exposure to H_2O_2 (oxidative stress). HSP60 was chosen as it

was revealed in Chapter 2 to correlate positively with MLSP in isolated mitochondria, and has been shown to correlate positively with MLSP at the level of whole tissues (Salway et al., 2011a). Therefore, I wanted to determine whether this relationship was also observed in a single cell type and the extent to which its induction by stress correlates with MLSP. GRP78 was also chosen for this study as this protein is essential to the unfolded protein response in the endoplasmic reticulum (major site of protein synthesis). Furthermore, it has been shown previously that GRP78 levels are positively correlated to MLSP in brain, heart, and liver tissues in 13 mammalian and avian species (Salway et al., 2011a). While other HSPs would be beneficial to include to get a more complete picture of the role HSPs have in establishing MLSP (such as the major cytosolic HSP, HSP70), time constraints limited the study to these two.

To investigate the relationship between the levels of these two chaperones and MLSP, myoblasts from 10 mammalian species (MLSP varying from 4 years to 122 years) were cultured at physiological oxygen levels (3%) and Western blots were performed, using antibodies to regions that are 100% conserved amongst all species included, to measure basal levels of HSP60 and GRP78. I extended this investigation to include stress induction of the above mentioned proteins by inducing oxidative stress through the administration of H_2O_2 and measuring the resulting increases in the levels of these two HSPs. For the reasons discussed in Chapter 2, all results were analyzed using residuals to remove any confounding effects of body mass.

3.3 Materials and Methods

3.3.1Materials

Dulbecco's Modified Eagle Medium (DMEM) with Earl salts, L-glutamine and sodium bicarbonate, DMEM with high glucose, L-glutamine and sodium bicarbonate, and DMEM: Nutrient Mixture F-12 were obtained from Sigma-Aldrich (Oakville, ON, Canada). Penicillin/streptomycin, ampicillin, gentamicin, non-essential amino acids, fetal bovine serum, and trypsin were purchased from Hyclone (Logan, UT, USA). All other chemicals were purchased from Bioshop (Burlington, ON, Canada), Fisher Scientific (Mississauga, ON, Canada) and Sigma Aldrich (Oakville, ON, Canada). BioRad protein dye was purchased from BioRad Laboratories (Hercules, California, USA). Anti-Hsp60 (ab46798), anti-GRP78 (ab21685) primary antibodies were purchased from Abcam (Cambridge, Massachusetts, USA). Anti-beta actin (sc-47778) primary antibody was purchased from Santa Cruz (Dallas, TX, USA). Anti-rabbit and anti-goat secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, US). Prestained broad range protein marker was obtained from BioLabs, (New England, Massachusetts, USA). Pierce Memcode reversible protein stain was purchased from Thermo Scientific (Rockford, Illinois, USA).

3.2.2 Cell Culture: Myoblast Isolation

Myoblast cells lines were established previously by another member of the lab (Robb et al., 2012). To isolate primary myoblasts, hind limb skeletal muscle was excised from healthy, young adults. Cow, pig, goat, and rabbit were all obtained from local abattoirs. Rat, gerbil, guinea pig and mice were ordered from Charles River, MA. Human myoblast cell lines were

purchased from Cell Applications Inc (see Table 3.1). All muscle tissue was excised at the time of death and kept on ice in sterile PBS with streptomycin, ampicillin, penicillin, and gentamicin until all connective tissue, blood vessels, and fat were removed. Myoblasts were isolated following a modified procedure from Barani et al. (2003). Briefly, muscle tissue was finely minced and suspended in Pronase (2mg/mL) at 37^0C. The muscle suspension was triturated every 15 minutes for 1 hour, after which it was filtered through sterile cheesecloth and centrifuged at 240g for 5 minutes. The resulting supernatant was then centrifuged at 500g for 5 minutes, after which, the pellet was resuspended in growth media (Ham's/F-10 nutrient mixture containing 20% FBS, non-essential amino acids, penicillin, streptomycin, gentamicin, amphotericin, and basic human growth factor). The suspension was then plated onto a collagen coated tissue culture dish and incubated in a Thermo Scientific Forma® Series II HEPA Class 100 water jacketed CO_2 incubator at 37^0C, 3% O_2, and 5% CO_2 . After 48 hours, an equal volume of media (DMEM with high glucose, l-glutamine, and sodium bicarbonate, 10% FBS, non-essential amino acids, penicillin, streptomycin, and basic human growth factor) was added and changed at 96 hours after plating. Myoblasts were evident after four days. At approximately 70% confluence, cells were washed with 5 ml sterile PBS, and 3 mL of trypsin was added for 3 minutes. After this time, 5 mL of growth media (DMEM with high glucose, l-glutamine, and sodium bicarbonate, 10% FBS, non-essential amino acids, penicillin, streptomycin, and basic human growth factor) was added, and the resulting suspension was centrifuged at 240g for 3 minutes. The resulting pellet was then resuspended in 1.5 mL of growth media, placed in a cryovial, and stored in a Nalgene "Mr. Frosty" freezing container overnight at -80^0C before being placed in a Taylor-Wharton High Capacity 35 cryogenic refrigerator.

Table 3.1: Body mass and MLSP data for the mammalian species included in this study.

Species MLSP data are from AnAge (de Magalhaes et al., 2005).

Species (common name)	Species (scientific name)	Number of Individuals	MLSP	Body Mass (g)
Syrian hamster	*Mesocricetus auratus*	4	3.9	105
Mouse	*Mus musculus*	3	4.0	20
Norway rat	*Rattus norvegicus*	4	4.5	300
Mongolian gerbil	*Meriones unguiculatus*	3	6.3	53
Rabbit	*Oryctolagus cuniculus*	5	9.0	1800
Guinea pig	*Cavia porcellus*	5	12.0	728
Cow	*Bos tarus*	2	20.0	750 000
Goat	*Capra hircus*	4	20.8	130 000
Pig	*Sus scrofa*	5	27.0	130 000
Human	*Homo sapiens*	2	122.0	62 035

3.3.3 Cell Culture: Basal Myoblasts

Myoblast cells lines were thawed and plated onto 10 cm diameter collagen coated tissue culture dishes with 10 mL of growth media (DMEM with high glucose, l-glutamine and sodium bicarbonate, 10% FBS, non-essential amino acids, penicillin and streptomycin) and incubated at 37^0C, 3% O_2, and 5% CO_2. At approximately 75% confluence, plates were split at a 1:3 ratio, using trypsin, following essentially the same procedure as for freezing down the isolated myoblasts. Cells were cultured in this manner until there were enough plates for collection (2 plates per individual). Cells were collected by washing the plates with 1 mL ice cold PBS, followed by harvesting using a disposable cell lifter. The resultant suspension was centrifuged (Thermo Scientific, IEC Micromax/Micromax RF) in a1.5 mL minifuge tube at 500 g for 5

minutes, the supernatant removed, and the pellet stored at -80^{0}C until required for further analysis.

3.3.4 Cell Culture: Stressed Myoblasts

Myoblast cell lines were maintained the in the same manner as the basal myoblasts for four passages until there were 16 plates for each individual cell line at approximately 75% confluence. Cells were then washed once with PBS and incubated in 10 mL serum free media for 18 hours prior to H_2O_2 exposure. The basic experimental design is shown in Table 3.2. At the start of the experiment, 2 plates were collected (in the same manner used to collect the basal myoblasts (above)) to serve as a baseline control (0 hr-control). At this time, 25µM H_2O_2 was added to 6 plates, and plates were placed back into the incubator. After 1 hour 2 plates exposed to H_2O_2 (1 hr-H_2O_2) and 2 unexposed plates (1 hr-control) were collected. This procedure was repeated 2 hours later (3-hr H_2O_2 and 3 hr-control). At this time, the remaining 2 plates exposed to H_2O_2 were rinsed with 1xPBS and 10 mL of fresh serum free media was added so that the cells could recover from H_2O_2 treatment (2 control plates were also rinsed and media switched to serve a control). This allows for the interpretation of how HSP60 and GRP78 levels change during the recovery phase following stress, and allows for adequate time for protein synthesis to occur (Ozawa et al., 2005). After 3 hours, the 2 plates that were exposed to H_2O_2 and rinsed (post-H_2O_2), the 2 unexposed plates (post-control), and the remaining 2 unexposed plates that were not rinsed (6 hr control) were collected. These conditions were chosen as they will induce oxidative stress without being toxic enough to cause cell death (Orzechowski et al., 2002; Caporossi et al., 2003; Wittstock et al., 2003). All cells were stored at -80^{0}C for further analysis.

Table 3.2: Conditions for the 8 time points used in this study. Each time point consisted of 2 plates of cells.

Time Point for Stress Exposure	Conditions for collection
0 hour start control	Media changed 18 hours prior to H_2O_2, and collected at the start of the experiment. No H_2O_2 exposure.
1 hour control	Media changed 18 hours prior to H_2O_2, and collected after 1 hour. No H_2O_2 exposure.
1 hour H_2O_2 exposure	Media changed 18 hours prior to H_2O_2. 25 uM H_2O_2 added, and cells collected after 1 hour.
3 hour control	Media changed 18 hours prior to H_2O_2, and collected after 3 hours. No H_2O_2 exposure.
3 hour H_2O_2 exposure	Media changed 18 hours prior to H_2O_2. 25 uM H_2O_2 added, and cells collected after 3 hours.
Recovery control	Media changed 18 hours prior to H_2O_2. Cells were left in incubator for 3 hours, at which point cells were rinsed with 1x PBS, the media was refreshed, and cells were collected after an additional 3 hours in the incubator. No H_2O_2 exposure.
Recovery after 3 hour H_2O_2 exposure	Media changed 18 hours prior to H_2O_2. 25 uM H_2O_2 added and cells were left in incubator for 3 hours. After 3 hours, the cells were washed with 1x PBS, the media was refreshed and the cells were collected after an additional 3 hours in the incubator.
6 hour end control	Media changed 18 hours prior to H_2O_2, and collected at the end of the experiment. No H_2O_2 exposure.

3.3.5 Preparation of Whole Cell Lysates

Cells were lysed by incubation for one hour in an equal volume of ice cold lysis buffer (10 mM Tris, pH 8.0, 150 mM sodium chloride, 2 mM EDTA, 2 mM DTT, 40% (v/v) glycerol, 0.4 mM phenylmethanesulfonylfluoride (PMSF), 40% (v/v) glycerol, and 0.5% (v/v) nonyl phenoxypolyethoxylethanol (NP-40)), and sonicated every 15 minutes (5 one second pulses followed by 5 seconds no sonication, repeated 3 times) (Sonicator W-375; Ultrasonic Inc., setting 5). After incubation, cell lysates were centrifuged at 10 000 g for 10 minutes at 4^0C

(Thermo Scientific, IEC Micromax/Micromax RF), and the supernatant was transferred into a new minifuge tube and stored at -80^{0}C until required for analysis.

3.3.6 Bradford Assay

Protein concentration in all lysates was determined using the same procedure as in Chapter 2.

3.3.7 CS Activity

CS activity was measured spectrophotometrically using a Bio-Tek PowerWave™ Microplate Scanning Spectrophotometer (KC4™ v3.4) with 96-well plates. Each well contained 0.5 mM DTNB, 0.1 mM AcoA, 0.005% Triton-X, all in 50 mM Tris (pH 8.0) (total volume of 100 uL), and 4 ug of whole cell lysate protein. Samples were heated to physiological body temperature, and the assay was run at 412 nm absorbance for 2 minutes to obtain any background signal. Following the initial run, 0.5 mM oxaloacetate was added to initiate the reaction, and enzyme activity was followed for 10 minutes. Specific activity was then calculated using the following equation: (slope/extinction coefficient (13.6/mMcm)*(optical path length/protein amount). Optical path was measured as the distance from the bottom of the well to the top of the well.

3.3.8 HSP60 and GRP78 Quantification

Whole cell lysate protein (10 ug) was separated by SDS-PAGE (4% stacking, 12% resolving gels) and electrotransferred to a PVDF membrane essentially as outlined in Chapter 2. To ensure even transfer, membranes were stained with Ponceau S reversible protein stain prior to

blocking and immunoblotting. After staining, membranes were blocked overnight (4°C) in a blocking solution that contained 5% skim milk in 1x phosphate buffer saline (PBS). Membranes were then probed for Beta-actin, HSP60 and GRP78 (using an antibody raised to an epitope that is 100% conserved across all species included in this study; figure 3.1). Anti-rabbit Beta-actin (1:200) primary antibody, anti-rabbit HSP60 (1:200) primary antibody, and anti-rabbit GRP78 (1:400) were added to 5% skim milk dissolved in 1x PBS-t and the membranes were incubated with antibody mixture for 1 hour at room temperature or overnight at 4°C. Membranes were then rinsed 5 times at 5 minute intervals with 1x PBS-t and probed with anti-rabbit secondary antibody for 1-2 hours at room temperature. Membranes were washed again with 1x PBS-t 4 times at 5 minute intervals, and once with 1x PBS for 5 minutes. Protein quantification was performed using the Odyssey infrared imaging system from LI-COR Biosciences, Version 1.0. For the basal myoblasts membranes, all HSP band intensities were standardized to actin, which served as a loading and transfer control in each lane. For between individual comparisons, all values were referenced to a single guinea pig sample that was run in each gel. All measurements were made in duplicate, with the location of each individual sample being randomly varied within the two gels. For the H_2O_2 exposure experiment, each membrane contained all 8 time points for one individual. Protein induction was determined by comparing the band intensity (standardized to beta-actin) for H_2O_2 exposed cells to time point-matched controls not exposed to H_2O_2. For HSP60 measurements, values were further standardized to CS activity to account for possible differences in mitochondrial content.

3.3.9 Statistical Analysis

Statistical analysis was performed using the same procedures as in Chapter 2.

HSP60 Sequence Alignment

Mouse	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Rat	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Hamster	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Rabbit	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Guinea Pig	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Gerbil	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Cow	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Pig	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR
Goat	----------GKSKFGADARALMLQGVDLLADAVAVTMGPKGR
Human	LTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR

GRP78 Sequence Alignment

Mouse	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Rat	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Hamster	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Rabbit	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Guinea Pig	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Gerbil	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Cow	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Pig	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Goat	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG
Human	EEEDKKEDVGTVVGIDLGTTYSCVGVFKNG

Figure 3.1: Sequence alignment for HSP60 and GRP78 for all mammals used in this study.

Partial sequence alignments for A) HSP60 and B) GRP78 showing the region in which amino acid sequence identity is 100% conserved and epitope (in red) to which the antibodies were raised. The epitope for beta-actin was not provided by the manufacturer, nor would they release that information. However, the amino acid sequence of this protein is 100% identical for all species included (see Appendix, Figure 4).

3.4 Results

3.4.1 MLSP is not correlated to HSP60 levels in cultured mammalian myoblasts at the basal level.

HSP60 was measured via Western blot in cultured myoblasts from 10 mammalian species. HSP60 levels were determined, standardized to beta actin (which served as a loading and transfer control) (Beta actin was not correlated to species MLSP (Appendix I, Figure 5)), an internal control (guinea pig), and the species aerobic capacity (CS activity, which does not correlate with species MLSP, Appendix 1, Figure 5) (Table 3.3). All data were natural logarithm (Ln) transformed and plotted against Ln-transformed MLSP data for each species (Figure 3.2A). A correlation analysis revealed a significant positive relationship between the HSP60 and MLSP only after the being standardized to aerobic capacity. To test whether this relationship was driven by body mass, residuals of body mass vs. MLSP (Appendix I, Figure 10) were plotted against the residuals of body mass vs. HSP60 levels (Figure 3.2 B). The residual analysis resulted in an insignificant correlation between MLSP and HSP60 levels, suggesting that MLSP is not correlated to HSP60 levels in mammalian myoblasts after accounting for differences in body mass.

Table 3.3. Non-transformed data for HSP60 levels (not standardized, standardized to beta actin (BA), and/or mitochondrial abundance (CS)) in cultured mammalian myoblasts from 10 different mammalian species. Each value is an average of between 2-6 individuals of each species, and standardized to an internal control (guinea pig). Standard error of the mean is also shown (SEM).

Species	MLSP	HSP60	SEM	HSP60/ BA	HSP60 /CS	HSP60 /CS/BA
Syrian hamster	3.9	0.660	0.170	0.765	0.00699	0.00817
Mouse	4.0	0.917	0.0521	2.29	0.00394	0.0100
Norway rat	4.5	0.742	0.0735	0.760	0.00914	0.00814
Mongolian gerbil	6.3	0.644	0.181	1.00	0.00615	0.00961
Rabbit	9.0	0.691	0.130	0.641	0.00451	0.00408
Guinea pig (GP)	12.0	0.659	0.0821	0.793	0.0118	0.0132
Cow	20.0	1.27	0.144	1.23	0.0147	0.0142
Goat	20.8	0.680	0.0980	0.984	0.00790	0.0113
Pig	27.0	0.802	0.164	0.880	0.0119	0.0129
Human	122.0	0.676	0.123	0.953	0.0146	0.0205

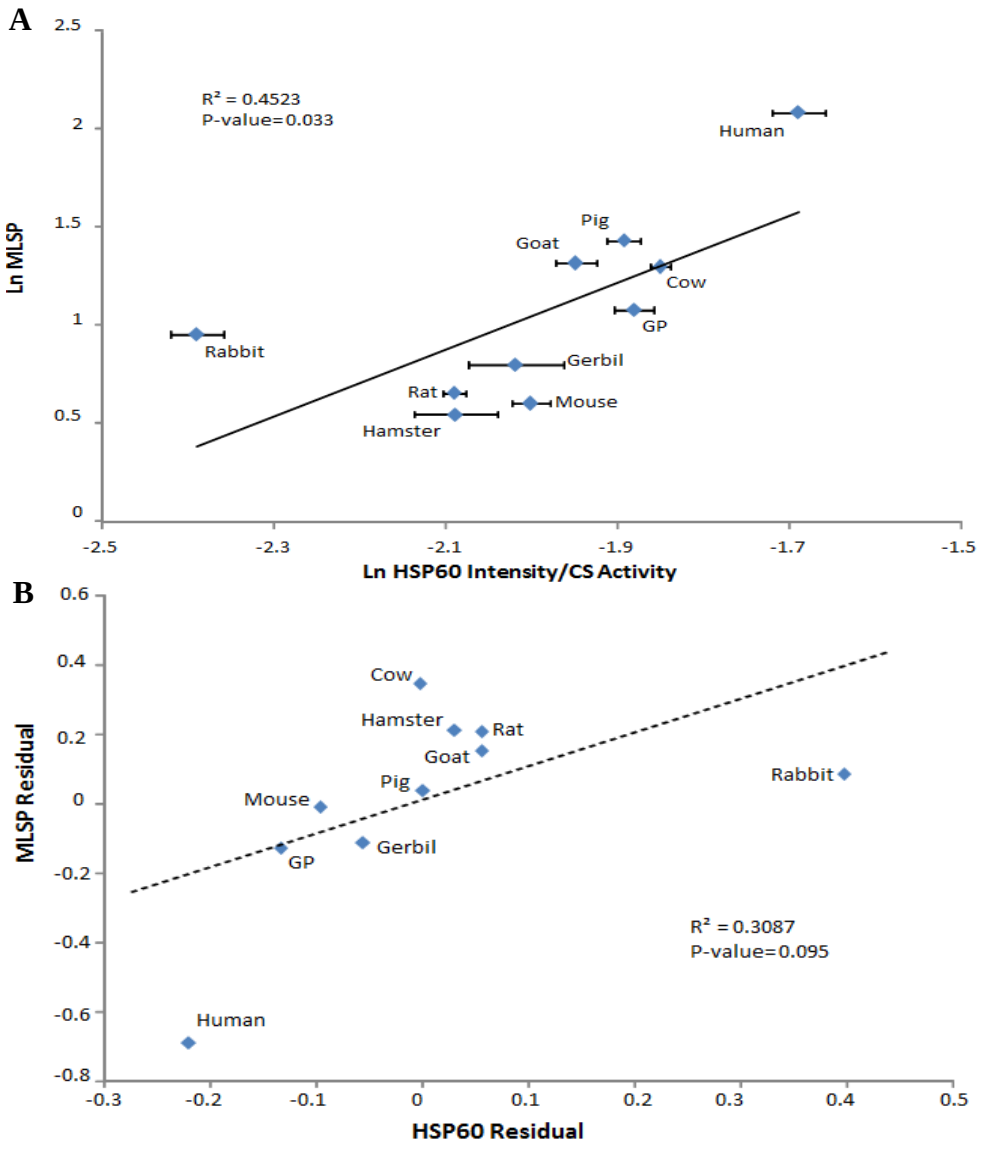

Figure 3.2: MLSP as a function of HSP60 levels in cultured mammalian myoblasts after standardization to beta actin and species aerobic capacity. A) MLSP is positively correlated to HSP60 levels. B) Residual analysis MSLP is not correlated to HSP60 levels. HSP60 levels were measured and standardized to the species aerobic capacity (measured as CS activity (µmol/min/mg)) for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual) and standardized to an internal control (guinea pig).

3.4.2 MLSP is not correlated to GRP78 levels in cultured mammalian myoblasts under basal conditions.

GRP78 levels were determined, standardized to beta actin (which served as a loading and transfer control), and an internal control (guinea pig) to minimize variability for comparisons between gels (Table 3.4). All data were natural logarithm (Ln) transformed and plotted against Ln-transformed MLSP data for each species (Figure 3.3A). No correlation was found between GRP78 levels and species. A residual analysis was performed (Figure 3.3B) to determine whether a correlation between MLSP and GRP78 was hidden by body mass, however this analysis also failed to reveal any significant correlation. Together, these data suggest that MLSP is not correlated to basal GRP78 levels in cultured mammalian myoblasts.

Table 3.4. Non-transformed data for GRP78 levels (not standardized, and standardized to beta actin (BA)) in cultured mammalian myoblasts from 10 different mammalian species.

Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM).

Species	MLSP	GPR78	SEM	GRP78/BA
Syrian hamster	3.9	0.818	0.0791	0.978
Mouse	4.0	0.525	0.0927	1.31
Norway rat	4.5	0.660	0.0473	0.647
Mongolian gerbil	6.3	0.728	0.101	1.20
Rabbit	9.0	0.690	0.0930	0.612
Guinea pig (GP)	12.0	0.626	0.0918	0.773
Cow	20.0	1.22	0.135	1.17
Goat	20.8	0.815	0.0953	1.14
Pig	27.0	1.06	0.0951	1.22
Human	122.0	0.650	0.130	0.923

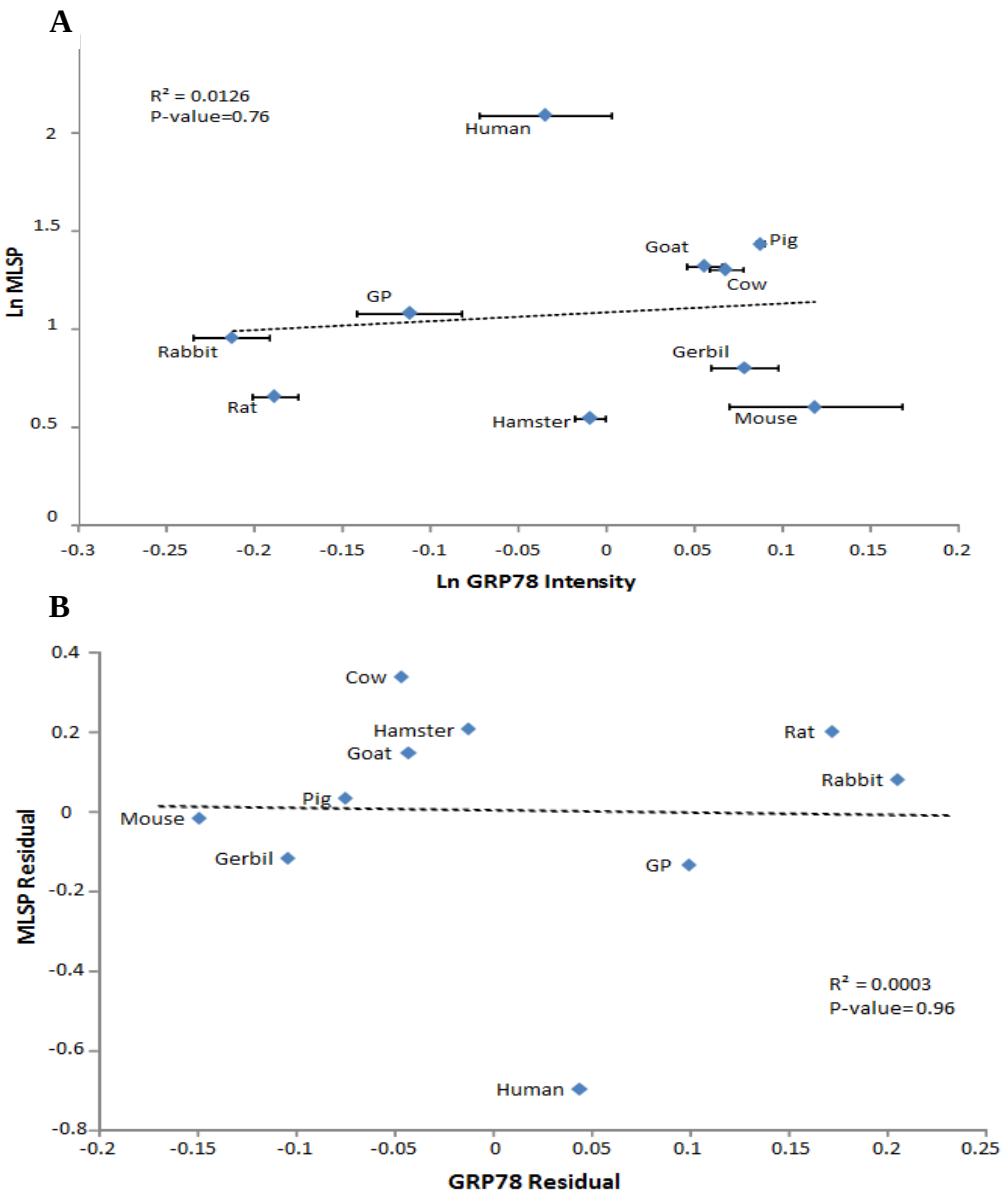

Figure 3.3: MLSP as a function of GRP78 levels in cultured mammalian myoblasts after standardization to beta actin. A) MLSP is not correlated to GRP78 levels. B) Residual analysis of MLSP is not correlated to GRP78. GRP78 levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual) and standardized to an internal control (guinea pig).

3.4.3 Stress induced HSP60 levels in cultured mammalian myoblasts are correlated to species MLSP but only after 1 hour H_2O_2 exposure.

HSP60 and GRP78 are both constitutively expressed and stress induced. To determine whether myoblasts from longer-lived mammalian species have a more robust induction of molecular chaperones following oxidative stress, cultured myoblasts from 10 mammalian species (2-5 individuals each) were exposed to H_2O_2 for 1 or 3 hours, and HSP60 levels were measured via western blot. HSP60 levels were determined for all 8 time points (see Table 3.2) and standardized to beta actin and species aerobic capacity (CS activity) (Table 3.5). All data were natural logarithm (Ln) transformed and plotted against Ln-transformed MLSP data for each species (Figure 3.4).

A correlation analysis revealed a positive correlation between MLSP and HSP60 induction after 1 hour H_2O_2 exposure (Figure 3.4A). To test whether this relationship was driven by body mass residuals of body mass vs. MLSP (Appendix I, Figure 10) were plotted against the residuals of body mass vs. HSP60 levels (Figure 3.5A). This analysis did not change the correlation between HSP60 induction and MLSP after 1 hour H_2O_2 exposure suggesting that HSP60 induction after 1 hour H_2O_2 exposure is higher in cultured myoblasts of longer-lived mammalian species. No correlation between HSP60 induction and MLSP was observed following 3 hour exposure to H_2O_2 or following a 3 hour recovery from 3 hour H_2O_2 exposure (Figure 3.4B and C; Figure 3.5B and C).

Table 3.5. Non-transformed data for HSP60 induction following H_2O_2 exposure in cultured mammalian myoblasts from 10 different species (standardized to beta actin (BA) and aerobic capacity (CS)). Each value is an average of between 2-6 individuals of each species. All values presented are a ratio of HSP60 levels from the H_2O_2 exposed cells to HSP60 levels in the time matched unexposed cells. For non-standardized data and standard error of the mean, see Appendix I, Table 3.

Species	MLSP	HSP60/BA/CS 1 Hour	HSP60/BA/CS 3 Hour	HSP60/BA/CS Post Recovery
Mouse	4.0	0.781	1.06	1.01
Norway rat	4.5	0.867	1.21	0.905
Mongolian gerbil	6.3	0.823	1.14	0.767
Rabbit	9.0	1.09	1.32	1.38
Guinea pig (GP)	12.0	0.970	1.52	1.30
Cow	20.0	1.15	1.59	1.29
Goat	20.8	1.26	0.954	1.66
Pig	27.0	2.07	1.56	1.00
Human	122.0	1.83	1.82	1.23

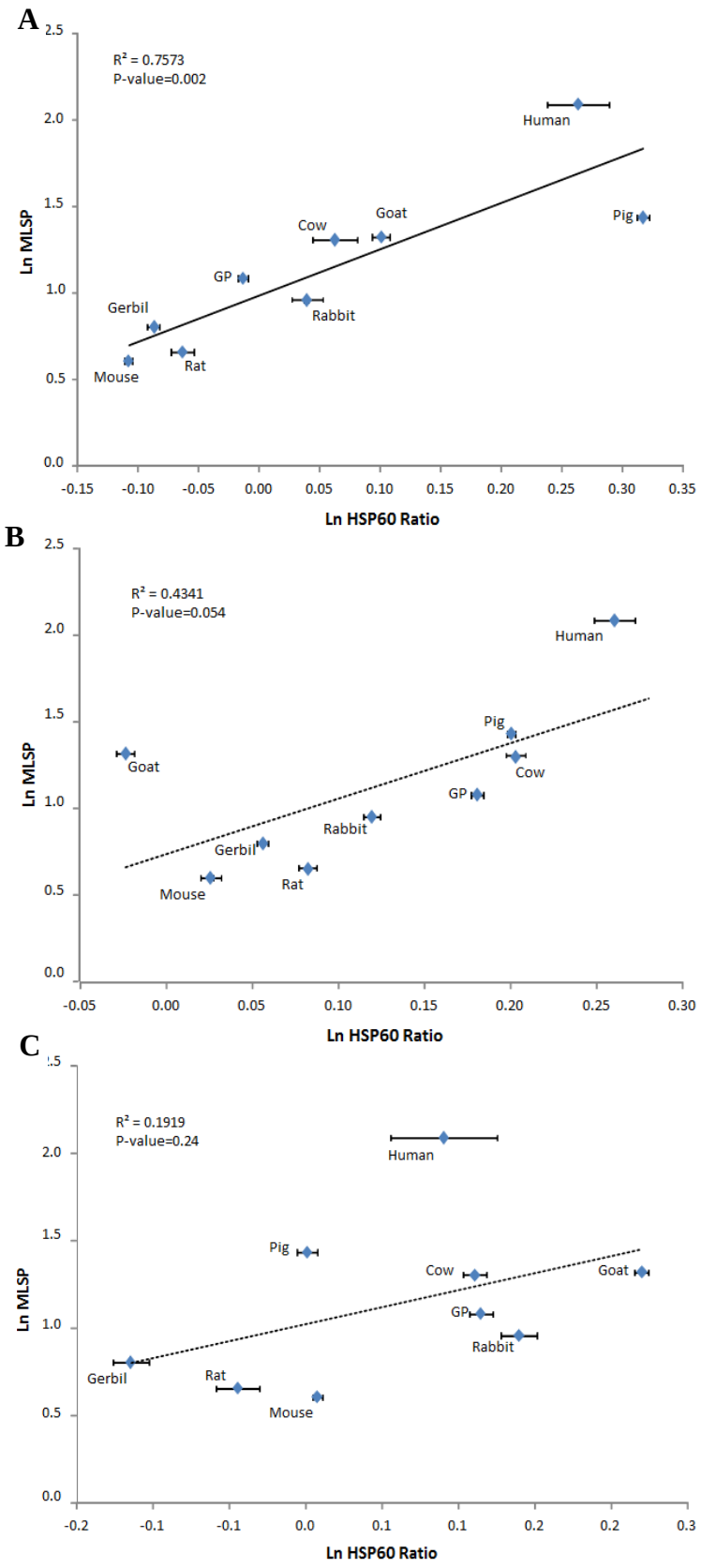

Figure 3.4: MLSP as a function of HSP60 induction following H_2O_2 exposure after standardization to beta actin and aerobic capacity. A) MLSP is positively correlated to HSP60 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to HSP60 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to HSP60 induction after 3 hour recovery from 3 hour H_2O_2 exposure. HSP60 levels were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the HSP60 levels from the H_2O_2 exposed cells by HSP60 levels in the time matched unexposed cells. Mitochondrial abundance was measured as CS activity (µmol/min/mg).

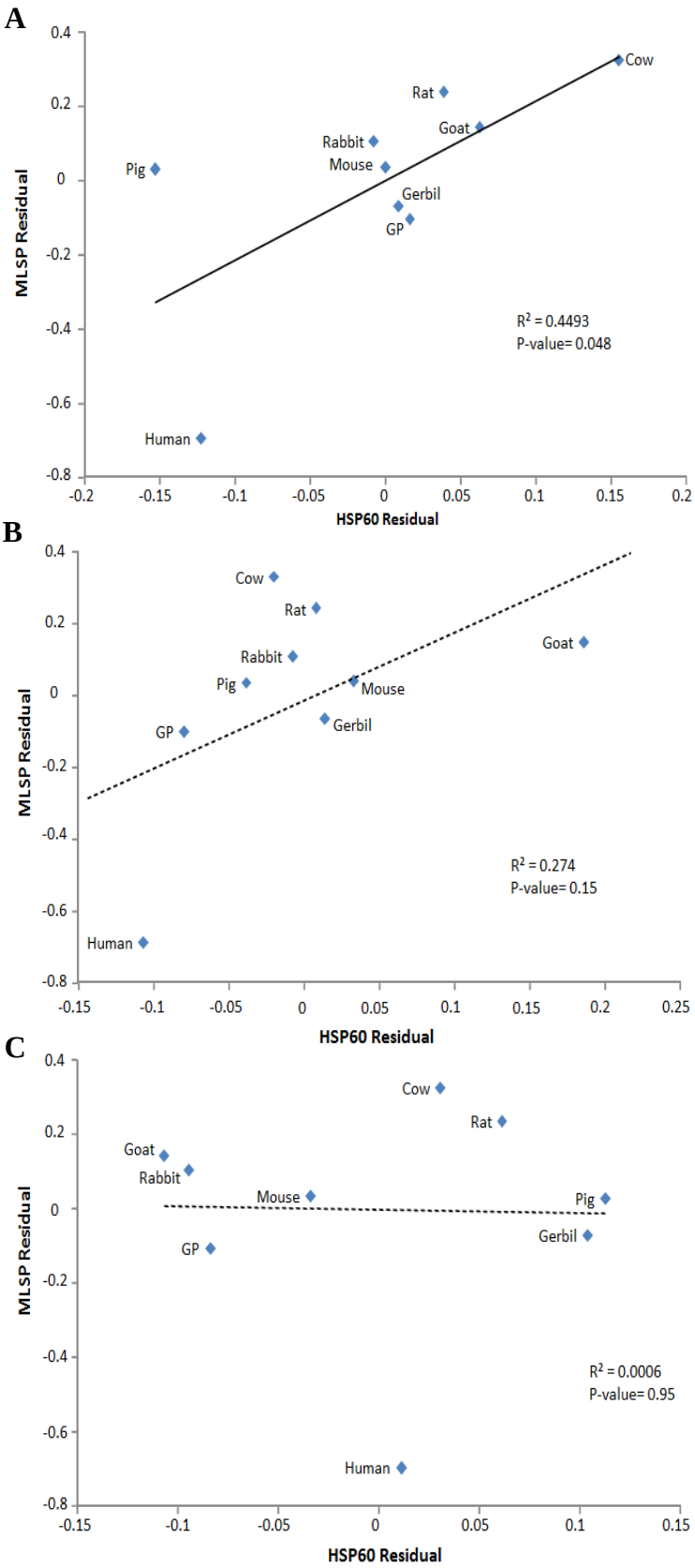

Figure 3.5: Residual analysis of MLSP as a function of HSP60 induction following H_2O_2 exposure after standardization to beta actin and aerobic capacity. A) MLSP is correlated to HSP60 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to HSP60 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to HSP60 induction after 3 hour recovery from 3 hour H_2O_2 exposure. HSP60 levels were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the HSP60 levels from the H_2O_2 exposed cells by HSP60 levels in the time matched unexposed cells. Mitochondrial abundance was measured as CS activity (µmol/min/mg).

3.4.4 Stress Induced GRP78 levels in cultured mammalian myoblasts are not correlated to species MLSP.

A correlation analysis revealed no significant relationship between GRP78 induction and MLSP after 1 hour H_2O_2 exposure, 3 hour H_2O_2 exposure, or after a 3 hour recovery period from H_2O_2 exposure (Figure 3.6). A residual analysis was performed to determine whether the correlations became significant when corrected for body mass (Figure 3.7), however this analysis did not change the correlation between GRP78 induction and MLSP after 1 hour exposure, 3 hour exposure, or after 3 hour recovery from H_2O_2. These data suggest that GRP78 induction following H_2O_2 exposure is not higher in cultured mammalian myoblast from longer-lived mammalian species.

It is important to note that MLSP data from AnAge were used in this study for all species included. This database reports the highest single observation of longevity for any given species. Due to a heavy bias for reporting of human lifespan data, the human MLSP estimate is likely biased toward increased longevity in this database. To account for this possibility, all analyses were repeated using a human MLSP of 90 years which, though somewhat arbitrary, nonetheless probably represents a fairer estimate within the comparative context (see Appendix II).

Table 3.6. Non-transformed data for GRP78 induction following H_2O_2 exposure in cultured mammalian myoblasts from 10 different species (standardized to beta actin (BA)). Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM). All values presented are a ratio of GRP78 levels from the H_2O_2 exposed cells to GRP78 levels in the time matched unexposed cells. For non-standardized data and standard error of the mean, see Appendix I, Table 4.

Species	MLSP	GRP78/BA 1 Hour	GRP78/BA 3 Hour	GRP78/BA Post Recovery
Mouse	4.0	0.944	0.718	1.20
Norway rat	4.5	1.12	0.776	1.07
Mongolian gerbil	6.3	0.917	0.962	0.981
Rabbit	9.0	1.19	1.07	0.872
Guinea pig (GP)	12.0	1.01	0.998	1.23
Cow	20.0	1.90	1.97	1.59
Goat	20.8	1.18	1.16	1.44
Pig	27.0	1.52	1.07	1.00
Human	122.0	1.39	1.32	1.80

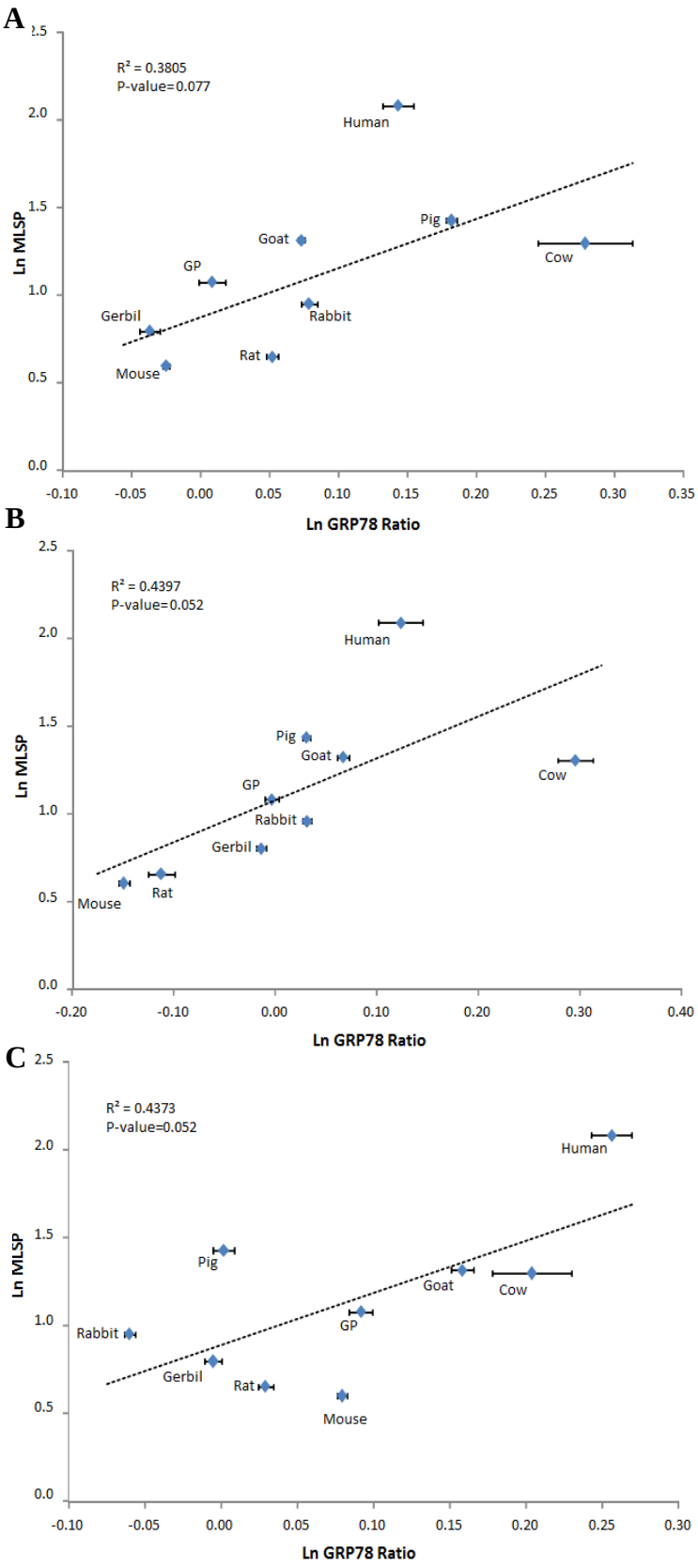

Figure 3.6: MLSP as a function of GRP78 induction following H_2O_2 exposure after standardization to beta actin. A) MLSP is not correlated to GRP78 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to GRP78 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to GRP78 induction after 3 hour recovery from 3 hour H_2O_2 exposure. GRP78 levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the GRP78 levels from the H_2O_2 exposed cells by GRP78 levels in the time matched unexposed cells.

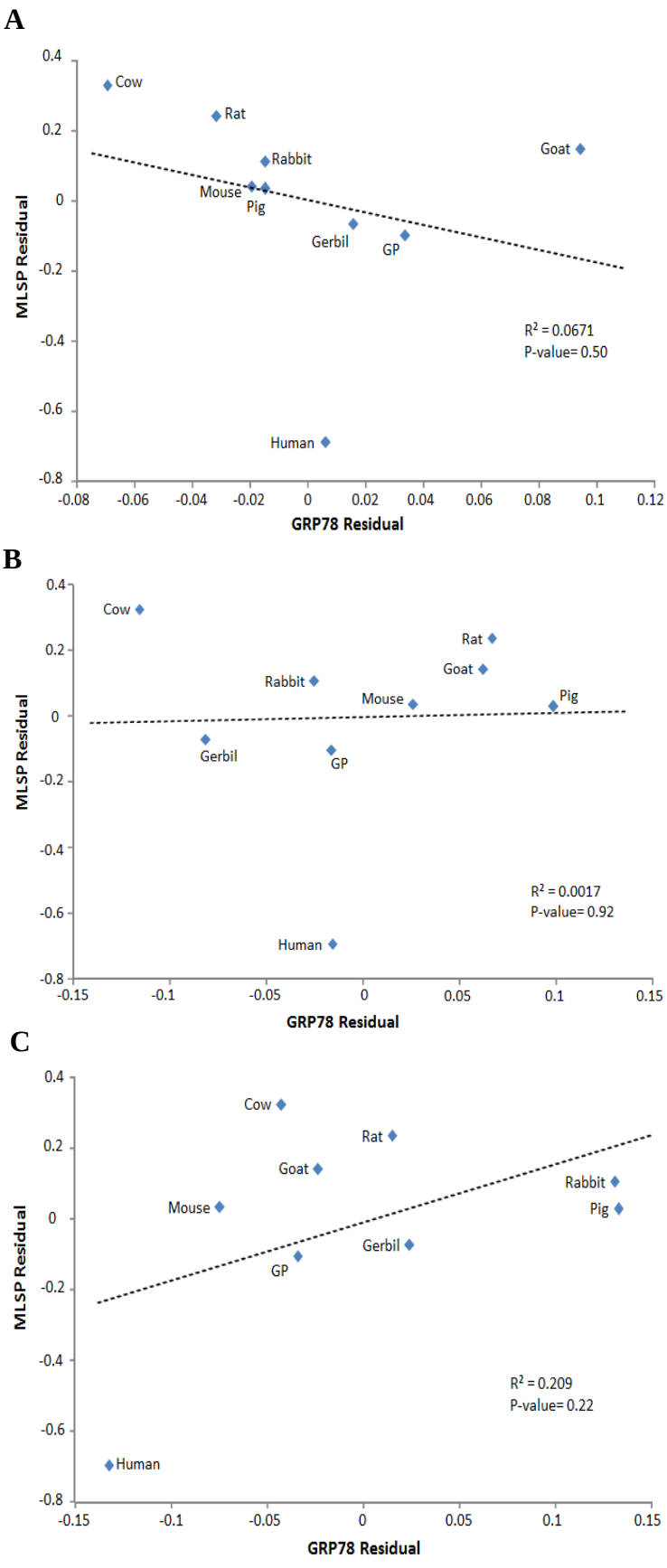

Figure 3.7: Residual analysis MLSP as a function of GRP78 induction following H_2O_2 exposure after standardization to beta actin. A) MLSP is not correlated to GRP78 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to GRP78 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to GRP78 induction after 3 hour recovery from 3 hour H_2O_2 exposure. GRP78 levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the GRP78 levels from the H_2O_2 exposed cells by GRP78 levels in the time matched unexposed cells.

3.5 Discussion

In Chapter 2 it was determined that HSP60 levels in isolated brain mitochondria are positively correlated with species MLSP. However, this measurement was made in whole brain tissue harvested from animals that had not been intentionally subjected to stress, and therefore do not provide any insights into which individual cell types might be important. Furthermore, only the basal level of HSP60 could be quantified in Chapter 2 as there was no way to test the induction of HSP60 following stress in brain tissue, which could be important in the context of longevity. While it would have been beneficial to use cultured neurons in this study (which would have provided insight into what individual brain cells are important in determining MLSP), growing adult neurons in culture is extremely difficult as they have low replicative ability. Therefore, myoblasts were used in this study as muscle tissue also experiences degeneration with age. The use of isolated myoblasts allows for the study of a single cell type maintained under identical conditions, and also provides the ability to measure the cells response to oxidative stress (induced by the addition of H_2O_2).

As observed by Salway et al (2011a), and in isolated brain mitochondria (Chapter 2), HSP60 levels in myoblasts were positively correlated with species MLSP. Together, these data strongly indicate that higher basal levels of HSP60 are associated with longevity. However, data from this study show that there is no correlation of basal HSP60 levels and MLSP. Following H_2O_2 exposure, it was determined that the magnitude of HSP60 induction correlated with species MLSP, however this correlation was only observed after 1 hour exposure. The data failed to reveal any correlation with HSP60 induction following 3 hour H_2O_2 exposure, or following a 3 hour recovery from H_2O_2 exposure. This suggests that perhaps the initial response to stress is the most important; however this hypothesis remains to be determined.

HSP60 induction is under the control of the heat shock factor (HSF) family of transcription factors. There are four members of the HSF family, and all of them regulate HSP expression, with HSF-1 as the primary HSF responsible for transcription of HSPs in response to cellular stress (Morley et al., 2004; Hsu et al., 2003). During times of stress, HSF-1 will bind to heat shock elements (which are upstream of the HSP gene) and stimulate transcription. Since HSF-1 is the prominent transcription factor for HSP transcription during stress, it is possible that HSF-1 protein levels are higher in longer-lived species, which could account for the greater induction of HSP60 after 1 hour of H_2O_2 exposure due to a higher availability of HSF-1 to induce transcription. Additionally, it is possible that this increased induction could also be explained by higher levels of enzymes involved in the activation of HSF-1. Activation of HSF-1 is a multi-step process which involves phosphorylation, dephosphorylation, acetylation, and deactelylation steps (reviewed in Calderwood et al., 2009). For example, HSF-1 can be acetylated at lysine residues, leading to inactivation. Therefore, the higher induction of HSP60 after 1 hour stress exposure could be explained by higher activities of proteins involved in post-translational modifications that activate HSF-1. However, whether the activities of these proteins correlate with MLSP remains to be determined.

In Chapter 2 it was suggested that the correlation between HSP60 levels and MLSP was due to increased levels of IGF-1 in the shorter-lived species. While this relationship could also be true for myoblasts *in vivo*, in this study the effect of IGF-1 on HSP levels is irrelevant, as the external environment for all cells included in this study were uniform and had the equal amounts of growth factors (including IGF-1). It is possible that no difference for HSP60 levels under basal conditions were observed due to an incompatibility between the growth factors present in the donor serum (required for cell culture) and the receptors for those growth factors on the cell

membrane of each species. However, a study done that looked at the effect of donor serum on metabolic rate in cultured myotubes (which differentiate from myoblasts) found no evidence for incompatibilities (Robb et al., 2012). The absence of an effect of donor serum on cultured myoblasts *ex vivo* also suggests that the correlation of an induction of HSP60 following 1 hour H_2O_2 exposure and MLSP is not a consequence of IGF-1 signalling.

GRP78 levels also did not correlate with species MLSP under basal conditions, which contradicts previous findings of a relationship between GRP78 levels in whole brain, liver, and heart tissues of endothermic vertebrate species (Salway et al., 2011a). This suggests that the correlation between MLSP and GRP78 levels observed in tissue homogenates might not be based on genetic differences, but rather on differences in the environments that the animals were exposed to. Furthermore, GRP78 induction also failed to reveal a significant correlation with MLSP which strengthens the argument for a lack of correlation between GRP78 and longevity. One explanation which could explain a lack of significant induction of GRP78 following oxidative stress could be due to the fact that GRP78 is regulated by multiple transcription factors which bind to an ER stress response element (ERSE) on the GRP78 gene to induce transcription. These factors include NF-Y, YY1, TFII-I and ATF6 (which is an ER transmembrane protein). During times of stress, ATF6 becomes cleaved and the nuclear portion will relocate to the nucleus, interact with YY1, and bind to the ERSE to initiate transcription (Baumeister et al., 2005). Due to the complexity of GRP78 transcription, it is possible that 3 hours of H_2O_2 exposure (and a total of 6 hours for the whole experiment) was not enough to fully induce transcription and translation, and as a result, no induction would be observed. While data on GRP78 induction is limited, one study did observe an increase in GRP78 mRNA levels following 20 µM H_2O_2 exposure in myoblast cell lines, however this was only observed after 24

hours of exposure, and the peak induction of GRP78 did not occur until 6 hours into the experiment (Ozawa et al., 2005).

In conclusion, data from this study provide equivocal support for a role of protein homeostasis in determining MLSP. There is no correlation between GRP78 levels at the basal levels or after being induced by oxidative stress. Similarly, HSP60 levels also fail to correlate with MLSP at the basal level. However, longer-lived species do have a greater induction of HSP60 after 1 hour exposure to oxidative stress, but not after 3 hour exposure or a 3 hour recovery. Together, these data provide some evidence for a role of molecular chaperones in determining MLSP, but due to the limited number of molecular chaperones studied, further studies should be done to provide further evidence for, or against, a role of molecular chaperone induction following oxidative stress in cultured myoblasts.

Chapter 4: General Discussion

According to the mitochondrial free radical theory of aging (MFRTA; Harman, 1972), aging in organisms arises due to accumulated cellular damage as a result of free radical production in mitochondria. This theory is based on observations of increased ROS production and macromolecular damage with age, increased mitochondrial dysfunction mitochondria with age, and the observation that many of the diseases associated with aging are characterized by dysfunctional mitochondria (Baba et al., 1998; Schapira et al., 1990; Lin et al., 2006). There are two testable predictions that arise from the MFRTA which pertain to this thesis. First, longer-lived species should have higher levels and activities of proteins involved in ROS neutralization as this would slow the rate of macromolecular damage. Second, long-lived species should also show increased mechanisms to repair damaged macromolecules (specifically proteins in this thesis) thereby delaying their accumulation.

In mitochondria, there are numerous antioxidant enzymes that aid in the removal of ROS. Once the superoxide anion is converted to H_2O_2 by spontaneous dismutation or by MnSOD, other antioxidant enzymes act to remove H_2O_2. One system that is involved in H_2O_2 removal is the mitochondrial GSH system. This system is important in maintaining cellular redox homeostasis and may potentially be an important determinant of lifespan. Overexpression of GPx can increase stress resistance in mice (Dabkowski et al., 2008; Lie and Cheng, 2005; Ran et al., 2006) and lifespan in *D. melanogaster* (Mockette et al., 1999) suggesting that GPx activity (and GR activity which is required for maintaining a pool of reduced GSH might be related to longevity. However, my measurements of the activities of GPx and GR in isolated brain mitochondria from 7 mammalian and 2 avian species indicate no relationship between MLSP

and either enzyme and therefore do not support the first prediction of the MFRTA. This result is similar to those observed in studies of antioxidant enzymes in whole tissues isolated from brain, liver, and heart (Page et al., 2010) (though in that study only the total cellular activities of GPx and GR were quantified) and in cultured mammalian fibroblasts from eight different endothermic vertebrate species (Brown et al., 2007) .

Studies of antioxidant enzyme activities in long-lived mutant mice with decreased IGF-1 signalling similarly challenge the MFRTA. For example, one study found increased catalase activities in liver, brain, and skeletal muscle tissues of *Irs1*$^{-/-}$ (insulin receptor substrate 1 null) mice (deficient in IGF-1 signalling), but no differences in CuZnSOD, MnSOD, GPx, or GR in these same tissues (Page et al., 2013). However another study observed no difference in CAT activities in brain and heart tissue of Snell dwarf mice (also deficient in IGF-1 signalling), and increased GPx activity in heart tissue of (Page et al., 2010). Similarly, the Ames dwarf mouse exhibits increased activity of CAT in liver and kidney tissues, increased GR activity in heart tissue, but decreased GPx activity in kidney and heart tissue (Brown-Borg et al., 1999). Based on these results, it cannot be concluded that antioxidant enzymes are responsible for the extended lifespan observed in these long-lived mutant mice. Similarly, transgenic experiments in which antioxidant enzymes are globally upregulated in mice reveal no consistent effect on lifespan (reviewed in Perez et al., 2009). Taken together, these data provide little evidence for a correlation between antioxidant enzymes and lifespan in endothermic vertebrates, and as such, challenge the MFRTA.

The second prediction arising from the MFRTA is that longer-lived species should have higher levels and/or activities of proteins involved in repairing damaged macromolecules. In this thesis, the hypothesis that longer-lived species have higher levels of molecular chaperones was

tested. Protein homeostasis appears to be a critical component of longevity particularly in brain tissue where protein aggregation is a characteristic of many neurodegenerative diseases (Kimura et al., 2007; McArdle et al., 2004; McLean et al., 2002; Morrow et al., 2010). To prevent protein aggregation, various cellular mechanisms promote refolding damaged proteins back to their native state. The importance of molecular chaperones (and HSPs) in longevity has been illustrated in *C. elegans*, in which overexpression of HSP-6 (Yokoyama et al., 2002) and HSP-16 (Walker et al., 2003) increases lifespan. Further support for a role of HSPs in aging arises from studies on *D. melanogaster* in which lifespan extension results from overexpression of HSP-22 or HSP-27 (reviewed in Morrow et al., 2010). Additionally, levels of HSP60, HSP70, and the endoplasmic reticulum molecular chaperones, GRP78 and GRP94, correlate positively with species MLSP in brain, heart, and liver tissue from 14 different mammalian and avian species (Salway et al., 2011a). All these data suggest that molecular chaperones are important in establishing protein homeostasis and can promote longevity.

HSP60 is the major HSP in mitochondria, however there is evidence that this protein is also found in the cytosol and in some cells also the outer leaflet of the plasma membrane (Gupta et al., 2002). Therefore, while HSP60 was previously shown to be correlated to species MLSP in brain tissue, it is possible that this finding was confounded by non-mitochondrial HSP60, and did not reflect mitochondrial HSP60 levels. Therefore, HSP60 levels were measured in isolated brain mitochondria and results from this study (Chapter 2) also revealed a positive correlation with MLSP, suggesting that HSP60 is indeed involved in the aging process. However, as previously mentioned in Chapter 2, HSP60 transcription is inhibited by IGF-1, and that IGF-1 levels are negatively correlated to body mass. Therefore it is possible that the correlations observed for HSP60 are an indirect consequence of IGF-1 levels, however there is no empirical evidence on

whether this is indeed the case. It would have been interesting to measure IGF-1 levels concurrently with HSP levels to determine whether this positive correlation observed here remained after being corrected for IGF-1 levels. Regardless, the results presented in this study are equivocal and cannot confirm or deny a role for protein homeostasis in longevity.

It is interesting to note that IGF-1 is primarily responsible for growth and development. Therefore, smaller species with higher levels of IGF-1 will also show a faster growth rate. Indeed, when looking at growth rates of species, smaller species generally grow faster, reach sexual maturity faster, and die sooner (de Magalhães and Costa, 2009). The evolutionary theories of aging predict that increased adult mortality leads to a decreased MLSP. The majority of research done on the evolutionary theories of aging analyzes lifespan through three life history characteristics that include adult mortality rate, age of maturity, and annual fecundity. Species that experience high predation will have an increased adult mortality rate, and thus an earlier age of maturity and a higher annual fecundity (Charnov, 1993). Therefore, species that experience higher predation rates must also grow and develop faster to reach sexual maturity before they are consumed by predators, in order to ensure that they produce offspring to keep the species alive. It seems fitting that smaller species have higher predation levels, and thus must grow faster. As a consequence of growing faster, they must also have elevated levels of IGF-1, which would result in the observed lower levels of HSPs.

Surprisingly, basal HSP60 levels in cultured mammalian myoblasts do not correlate with MLSP, nor do basal GRP78 levels. However, following H_2O_2 exposure, longer-lived speceis show a greater induction of HSP60 levels after 1 hour exposure, but not after 3 hour exposure of a 3 hour recovery, suggesting that a rapid initial response to oxidative stress is important. GRP78 levels did not correlate with species MLSP following H_2O_2 exposure. Thus, the effect

seems to be confined to mitochondrial HSP60. Together, these data provide equivocal evidence for a role of molecular chaperones in determining MLSP.

The increase in HSP60 levels following H_2O_2 exposure is consistent with the role of this protein in the cellular stress response to negate the damages caused to mitochondrial proteins. However, the mechanisms that cause this increase in HSP60 remain to be determined. HSP60 is under the control of HSF-1, the primary factor responsible for transcription of HSPs in the cellular stress response. When HSPs are needed for protein folding, HSF-1 will localize to the nucleus where it is becomes active after being hyperphosphorylated (Holmberg et al., 2001; Westerheide et al., 2009). It is possible that the higher induction of HSP60 after 1 hour H_2O_2 exposure in the long-lived species could be caused by higher activities of enzymes involved in these post-translational modifications of HSF-1 resulting in a faster activation of HSF-1 and quicker transcription of the HSP60 gene. Therefore, the increased HSP60 induction seen in longer-lived species could be explained due to increased availability and activity of enzymes responsible for these modifications; however, to date there is no information on the levels of these enzymes and their correlation with species MLSP.

In concordance with the equivocal results on protein repair mechanisms, other mechanisms of damaged macromolecular repair also fail to reveal any significance to the aging process. Activities of glutaredoxin and thioredoxin (which aid in removing oxidative insults from proteins) do not correlate with species MLSP. Furthermore, the activities of these proteins were marginally lower in Snell dwarf mice compared to normal mice (Salway et al., 2011b), and glutaredoxin activity in the naked mole rat was similar to the activity of the common mouse (Perez et al., 2009a). In addition, the major cytosolic proteasomes (which degrade damaged proteins) failed to correlate with MLSP, and the activities of these proteins were actually lower

in long-lived Snell dwarf mice when compared to normal WT mice (Salway et al., 2011b). Similar results were also observed with respect to DNA repair enzymes. In a cross-species comparison, the activities of two enzymes involved in base excision repair (which removes single nucleotides that are damaged due to oxidation or alkylation (see Barnes and Lindahl, 2004 for review)), abasic endonuclease and DNA polymerase β, revealed no correlation to MLSP in brain and liver tissues of 15 vertebrate endotherm species (Page et al., 2012), or in cultured dermal fibroblasts from nine mammalian species (Brown et al., 2007). Taken together, the data in the literature are ambiguous therefore challenge the second prediction of the MFRTA.

If the MFRTA is correct then my results should have been different; I would predict that I should have seen higher levels of antioxidant enzymes and molecular chaperones in the longer-lived species, and a greater induction of HSP60 and GRP78 following exposure to oxidative stress for all time points. Therefore, data from the this thesis and the literature provide equivocal evidence for the MFRTA, suggesting that its role in aging is not as clear cut as originally thought.

The MFRTA has been on the forefront of aging research for the past two decades. However, in light of many recent findings it is clear that the relationship between ROS formation and lifespan is a lot more complex than originally thought. Based on the results of this study, longer-lived species do not display increased GPx or GR activities, which is in agreement with many similar studies that look at many different antioxidant enzymes. Furthermore, while there is limited evidence presented in this thesis for increased protein repair, it is possible that the observed correlation between HSP60 and MLSP in brain mitochondria is due indirectly to IGF-1 levels. Moreover, molecular chaperones are responsible for folding newly synthesized proteins as well as correcting oxidative insults. Therefore, it is possible that this observed trend at the

basal level reflects a role for increased ability of proper folding of new proteins in establishing lifespan, rather than an increased ability to repair oxidatively damaged proteins. Taken together, the data in this thesis fail to provide enough evidence to fully support the MFRTA. Evidence from the literature also challenges this theory; however, there is some evidence that supports the MFRTA. Therefore, future research should continue to use the comparative method and focus on measuring potential correlates of longevity (especially HSPs at the basal level and after being induced) in a variety of cell types, under a variety of stress conditions to obtain a much more complete data set. Only then will researchers be able to obtain a clear picture of what molecular correlates are important in determining MLSP, and whether the MFRTA is the best explanation for why animals age.

References

Alam, Z.I., Jenner, A., Daniel, S.E., Lees, A.J., Cairns, N., Marsden, C.D., Jenner, P., Halliwell, B. (1997). Oxidative DNA damage in the Parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. *Journal of Neurochemistry*. 69(3):1196-1203.

Ali, Y.O., Kitay, B.M., Zhai, R.G. (2010). Dealing with misfolded proteins: examining the neuroprotective role of molecular chaperones in neurodegeneration. *Molecules*. 15:6859-6887.

Alway, S.E., Degens, H., Krishnamurthy, G., Smith, C.A. (2002). Potential role for Id myogenic repressors in apoptosis and attenuation of hypertrophy in muscles of aged rats. *American Journal of Physiology-Cellular Physiology*. 283:66-76.

Andersen, B., Pearse II, R.V., Jenne, K., Sornson, M., Lin, S., Bartke, A, Rosenfeld, M.G. (1995). The Ames dwarf gene is required for *Pit-1* gene activation. *Developmental Biology*. 172:495-503.

Anderson, G.L. (1978) Responses of dauer larvae of *Caenorhabditis elegans* (Nematoda:Rhabditidae) to thermal stress and oxygen deprivation. *Canadian Journal of Zoology.* 56:1786-1791.

Andziak, B., O'Conner, T.P., Qi, W., DeWaal, E.M., Pierce, A., Chaudhuri, A.R., Van Remmen, H., Buffenstein, R. (2006). High oxidative damage levels in the longest-living rodent, the naked mole rat. *Aging Cell.* 5:463-471.

Arantes-Oliveira, N., Apfeld, J., Dillin, A., Kenyon, C. (2002). Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*. *Science*. 295:502-505.

Austad, S.N. (1993). Retarded senescence in an insular population of Virginia opossums (*Didelphis virginiana). Journal of Zoology, London. 229*:695-708.

Azpurua, J., Yang, J., Seluanov, A. (2013). IGF1R levels in the brain negatively correlate with longevity in 16 rodent species. *Aging*. 5(4):304-314.

Baba, M., Nakajo, T., Pang-Hsien, T., Tomita, T., Nakaya, T., Lee, V.M.Y., Trojanowski, J.Q., Iwatsubo, T. (1998). Aggregation of a-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *American Journal of Pathology*. 152(4): 879-884.

Ballinger, S.W. (2005). Mitochondrial dysfunction in cardiovascular disease. *Free Radical of Biology and Medicin*e. 30(10):1278-1295.

Barja, G., Cadena, S., Rojas, R., Perez-Camp, R., Lopez-Torres, M. (2001). Low mitochondrial free radical production per unit O_2 consumption can explain the simultaneous presence of high longevity and high aerobic metabolic rate in birds. *Free Radical Research*. 21(5):317-328.

Barja, G. (2002). Rate of generation of oxidative-stress related damage and animal longevity. *Free Radical of Biology and Medicine*. 33(9):1167-1172.

Barsyte, D., Lovejoy, D.A., Lithgow, G.J. (2001). Longevity and heavy metal resistance in *daf-2* and *age-1* long-lived mutants of *Caenorhabditis elegans*. *Federation of American Societies for Experimental Biology Journal*. 15:627-634.

Bartke, A., Brown-Borg, H., Mattison, J., Kinney, B., Hauck, S., Wright, C. (2001). Prolonged longevity of hypopituitary dwarf mice. *Experimental Gerontology.* 36(1):21-28.

Baumeister, P., Shengzhan, L., Skarnes, W.C., Guangchao, S., Seto, E., Shi, Y., Lee, A.S. (2005). Endoplasmic reticulum stress induction of the Grp78/BiP promoter: Activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Molecular and Cellular Biology*. 25(11):4529-4540.

Belia, S., Pietrangelo, T., Fulle, S., Menchetti, G., Cecchini, E., Felaco, M., Vecchiet, J., Fano, G. (1998). Sodium nitroprusside, a NO donor, modifies Ca^{2+} transport and mechanical properties in frog skeletal muscle. *Journal of Muscle Research and Cell Motility*. 19:865-876.

Betarbet, R., Canet-Aviles, R.M., Sherer, T.B., Mastroberardino, P.G., McLendon, C., Kim, J.H., Lund, S., Na, H.M., Taylor, G., Bence, N.F., Kopito, R., Seo, B.B., Yagi, T., Yagi, A., Klinefelter, G., Cookson, M.R., Greenamyre, J.T. (2006). Intersecting pathways to neurodegeneration in Parkinson's disease effects of the pesticide rotenone on DJ-1 alpha-synuclein and the ubiquitin-proteasome system. *Neurobiology of Disease*. 22:404-420.

Bhardwaj, R.D., Curtis, M.A., Spalding, K.L., Buchholz, B.A., Fink, D., Bjork-Eriksson, T., Nordborg, C., Gage, F.H., Druid, H., Eriksson, P.S., Frisen, J. (2006). Neocortical neurogenesis in humans is restricted to development. *Proceedings of the National Academy of Science*. 103(33);12564-12568.

Bigot, A., Jacquemin, V., Debacq-Chainiaux, F., Butler-Browne, G.S., Toussaint, O., Furling, D., Mouly, V. (2008). Replicative aging down-regulates the myogenic regulatory factors in human myoblasts. *Biology of the Cell*. 100:189-199.

Bokov, A.F., Lindsey, M.K., Khodr, C., Sabia, M.R., Richardson, A. (2009). Long-lived Ames dwarf mice are resistant to chemical stressors. *Journal of Gerontology*. 64(8):819-827.

Brown, J.C., McClellan, G.B., Faure, P.A., Klaiman, J.M., Staples, J.F. (2009). Examining the mechanisms responsible for lower ROS release rates in liver mitochondria from the long-lived house sparrow (*Passer domesticus*) and big brown bat (*Eptesicus fuscus*) compared to the short-lived mouse (*Mus musculus*). *Mechanisms of Ageing and Development*. 130(8):467-76.

Brown-Borg, H.M. (1996). Longevity in mice: is stress resistance a common factor? *Age*. 28:145-162.

Calderwood, S.K., Murshid, A., Prince, T. (2009). The shock of aging: Molecular chaperones and the heat shock response in longevity and aging. *Gerontology*. 55:550-558.

Campisi, J. (2001). Cellular senescence as a tumor-supressor mechanism. *Trends in Cell Biology*. 11(11):27-31.

Cannon, J.R., Tapias, V., Na, H.M., Honick, A.S., Drolet, R.E., Greenamyre, J.T. (2009). A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of Disease*. 34:279-290.

Caporossi, D., Ciafre, S.A., Pittaluga, M., Savini, I., Farace, M.G. (2003). Cellular responses to H_2O_2 and bleomycin-induced oxidative stress n L6C5 rat myoblasts. *Free Radical Biology and Medicine*. 35(11):1355-1364.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., Partridge, L. (2001). Extension of lifespan by loss of CHICO, a *Drosophila* insulin receptor protein. *Science.* 292:104-106.

Coppe, J., Desprez, P., Krtolica, A., Campsisi, J. (2010). The senescence-associated secretory phenotype: The dark side of tumour suppression. *Annual Review of Pathological Mechanisms.* 5:99-118.

Coschigano, K.T., Clemmons, D., Bellush, L.L., Kopchick, J.J. (2000). Assessment of frowth parameters and lifespan of GHR/BP gene disrupted mice. *Endocrinology*. 141:2608-2613.

Crittenden, S.L., Eckmann, C.R., Wang, L.T., Bernstein, D.S., Wickens, M., Kimble, J. (2003). Regulation of the mitosis/meiosis decision in the *Caenorhabditis elegans* germ-line. *Philosophical Transactions of the Royal Society*. 358:1359-1362.

Csiszar, A., Labinskyy, N., Perez, V., Recchia, F.A., Podlutsky, A., Mukhopadhyay, P., Losonczy, G., Pacher, P., Austad, S.N., Bartke, A., Ungvari, Z. (2008). Endothelial function and vascular oxidative stress in long-lived GH/IGF-deficient Ames dwarf mice. *American Journal of Physiology: Heart and Circulatory Physiology*. 295(5):H1882-H1894.

Csiszar, A., Podlutsky, A., Podlutskaya, N., Sonntag, W.E., Merlin, S.Z., Philipp, E.R., Doyle, K., Davila, A., Recchia, F.A., Ballabh, P., Pinto, J.T., Ungvari, Z. (2011). Testing the oxidative stress hypothesis of aging in primate fibroblasts: is there a correlation between species longevity and cellular ROS production? *Journals of Gerontology Series A: Biological Sciences and Medical Science*. 67(8):841-852.

Darios, F., Corti, O., Lucking, C.B., Hampe, C., Muriel, M., Abbas, N., Gu, W, Hirsch, E.C., Rooney, T., Ruberg, M., Brice, A. (2003). Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. *Human Molecular Genetics*. 12(5):517-526.

de Magalhães J.P. (2006) Species selection in comparative studies of aging and antiaging research. In Handbook of Models for Human Aging. Elsevier Academic Press, MA. 9-20.

de Magalhães J.P., Costa J. (2009) A database of vertebrate longevity records and their relation to other life-history traits. *Journal of Evolutionary Biology*. 22:1770-1774.

Dabkowski, E.R., Williamson, C.L., Hollander, J.M. Mitochondria-specific transgenic overexpression of phospholipid hydroperoxide glutathione peroxidase (*GPx4*) attenuates ischemia/reperfusion-associated cardiac dysfunction. *Free Radical Biology and Medicine.* 45(6):855-865.

Dirks, A., Leeuwenburgh, C. (2001). Apoptosis in skeletal muscle with aging. *American Journal of Physiology-Regulatory, Integrative, and Comparative Physiology*. 282:519-527.

Eu, J.P., Sun, J., Xu, L., Stamler, J.S>, Meissner, G. (2000). The skeletal muscle calcium release channel: coupled O_2 sensor and NO signaling functions. *Cell*. 102:499-509.

Finch, C. E. (1990). *Longevity, Senescence, and the Genome*. The University of Chicago Press, Chicago and London.

Flurkey, K., Papaconstantinou, J., Miller, R.A., Harrison, D.E. (2001). Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proceedings of the National Academy of Science*. 98(12):6736-6741.

Flurkey, K., Papaconstantinou, J., Harrison, D.E. (2002). The Snell dwarf mutation Pit1(dw) can increase lifespan in mice. *Mechanisms in Ageing and Development.* 123:121-130.

Fridovich, I. (1983). Superoxide radical: an endogenous toxicant. *Annual Reviewsin Pharmocological Toxicity*. 23:239-57

Friedman, D.B., Johnson, T.E. (1988). A mutation in the *age-1* gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. *Genetic.* 118:75-86.

Fulle, S., Protasi, F., Di Tano, G., Pietrangelo, T., Beltramin, A., Boncompagni, S., Vecchiet, L., Fano, G. (2004). The contribution of reactive oxygen species to sarcopenia and muscle ageing. *Experimental Gerontology*. 39:17-24.

Garland T, Adolph SC. (1994) Why not to do two-species comparative studies: limitations on inferring adaptation. Physiol Zool. 67: 797-828.

Gifondorwa, D.J., Robinson, M.B., Hayes, C.D., Taylor, A.R., Prevette, D.M., Oppenheim, R.W., Caress, J., Milligan, C.E. (2007). Exogenous delivery of heat shock protein 70 increases lifespan in a mouse model of Amyotrophic Lateral Sclerosis. *Journal of Neuroscience*. 27:12173-13180.

Gimenez, L.E., Ghildval, P., Fischer, K.E., Hu, H., Ja, W.W., Eaton, B.A., WU, T., Austad, S.N., Ranian, R. (2013) Modulation of methuselah expression targeted to Drosophila insulin-producing cells extends life and enhances oxidative stress resistance. *Aging Cell*. 12(1):121-129.

Gogvadze, V., Orrenius, S., Zhivotovsky, B. (2008). Mitochondria in cancer cells: what is so special about them. *Trends in Cell Biology*. 18(4):165-173.

Gu, M., Gash, M.T., Mann, V.M., Javoy-Agid, F., Cooper, J.M., Schapra, A.H.V. (1996). Mitochondrial defect in Huntington's disease caudate nucleas. *Annals of Neurology*. 39(3):385-389.

Gupta, S., Knowlton, A.A. (2002). Cytosolic heat shock protein 60, hypoxia, and apoptosis.*Cirulation*. 106:2727-2733.

Halliwell, B. (2001). Role of free radicals in the neurodegenerative diseases. *Drugs and Aging*. 18(9):685-716.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of Gernotology*. 11:298-300.

Harman, D. (1972). The biologic clock: the mitochondria? *Journal of the American Geriatrics Society*. 20(4):145-147.

Harman, D. (1981). The aging process. *Proceedings of the National Academy of Science*. 78(11):7124-7128.][=-

Harper, J.M., Salmon, A.B., Leiser, S.F., Galecki, A.T., Miller, R.A. (2007). Skin-derived fibroblasts from long-lived mutant species are resistant to some, but not all, lethal stresses and to the mitochondrial inhibitor rotenone. *Aging Cell*. 6:1-13.

Harper, J.M, Wang, M., Galecki, A.T., Ro, J., Williams, J.B., Miller, R.A. (2011). Fibroblasts from long-lived bird species are resistant to multiple forms of stress. *The Journal of Experimental Biology.* 214:1902-1910.

Hauck, S.J., Bartke, A. (2000). Effects of growth hormone on hypothalamic catalase and Cu/Zn superoxide dismutase. *Free Radical of Biology and Medicine*. 28(6):970-978.

Hauck, S., Aaron, J.M., Wright, C., Kopchick, J.J., Bartke, A. (2002). Antioxidant enzymes, free-radical damage, and response to paraquat in liver and kidney of long-living growth hormone receptor/binding protein gene-disrupted mice. *Hormone Metabolism Research.* 34:481-486.

Heikkila, R.E., Hess, A., Duvoisin, R.C. (1984). Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. *Science*. 224:1451-1453.

Herrero, A., Barja, G. (1998). H_2O_2 production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. *Mechanisms of Ageing and Development*. 103:133-146.

Hsieh, C. DeFord, J.H., Flurkey, K., Harrison, D.E., Papaconstantinou, J. (2002). Implications for the insulin signaling pathway in Snell dwarf mouse longevity: a similarity with the *C. elegans* longevity paradigm. *Mechanisms of Ageing and Development*. 123:1229-1244.

Huang, L., Sun, G., Cobessi, D., Wang, A.C., Shen, J.T., Tung, E.Y., Anderson, V.E., Berry, E.A. (2005). 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation of complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. *The Journal of Biological Chemistry*. 281(9):5967-5972.

Jedlicka, P., Mortin, M.A., Wu, C. (1997). Multiple functions of *Drosophila* heat shock transcription factor *in vivo*. *European Molecular Biology Organization*. 16:2452-2462.

Johnson, T.E., Wood, W.B. (1982). Genetic analysis of life-span in *Caenorhabditis elegans. Proceedings of the National Academy of Science USA.* 79:6603-6607.

Johnson, T.E., Lithgow, G.J. (1992). The search for the genetic basis of aging: the identification of gerontogenes in the nematode *Caenorhabditis elegans. Journal of the American Geriatrics Society.* 40:936-945.

Kabil, H., Partridge, L., Harshman, L.G. (2007). Superoxide dismutase activities in long-lived *Drosophila melanogaster* females: chico1 genotypes and dietary dilution. *Biogerentology*. 8(2):201-208.

Kapahi, P., Boulton, M.E., Kirkwood, T.B.L. (1999). Positive correlation between mammalian lifespan and cellular resistance to stress. *Free Radical Biology and Medicine.* 26(5/6):495-500.

Kaplan, J. (2002). Spinocerebellar ataxias due to mitochondrial defects. *Neurochemistry International*. 40(6):553-557.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. *Nature.* 366:461-464.

Kimura K, Tanaka N, Nakamura N, Takano S, Ohkuma S.(2007). Knockdown of mitochondrial heat shock protein 70 promotes progeria-like phenotypes in *caenorhabditis elegans*. *Journal of Biological Chemistry.*282:5910–5918

Kirkwood, T.B.L., Austad, S.N. (2000). Why do we age? *Nature. 409*:233-238.

Kirkwood, T.B.L. (1977). Evolution of aging. *Nature.* 270:301-304.

Klass, M., Hirsch, D. (1976). Non-ageing developmental variant of *Caenorhabditis elegans. Nature.* 260:523-525.

Kregel, K.C., Zhang, H.J. (2007). An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *American Journal of Physiology*. 292(10): R18–R36.

Ku, H., Brunk, U.T., Sohal, R. (1993). Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radical of Biology and Medicine*. 15:621-627.

Kuroda, Y., Mitsui, T., Kunishige, M., Shono, M., Akaike, M., Azuma, H., Matsumoto, T. (2006). Parkin enhances mitochondrial biogenesis in proliferating cells. *Human Molecular Genetics*. 15(6):883-895.

Kuroso, H., Yamamoto M., Clark, J.D., Pastor, J.V., Nandi, A., Gurnani, P., McGuinness, O.P., Chikuda, H., Yamaguchi, M., Kawaguchi, H., Shimomur, I., Kakayama, Y., Herz, J., Kahn, C.R., Rosenblatt, K.P., Kuro-o, M. (2005). Suppression of aging in mice by the hormone klotho. *Science*. 309:1829-1833.

Lambert, A.J., Boysen, H.M., Buckingham, J.A., Yang, T., Podlutsky, A., Austad, S.N., Kunz, T.H., Buffenstein, R., Brand, M.D. (2007). Low rates of hydrogen peroxide production by isolated heart mitochondria associated with long maximum lifespan in vertebrate homeotherms. *Aging Cell*. 6:607-618.

Landry, J., Bernier, D., Chretien, P., Nicole, L.M., Tanguay, R.M, Marceau, J. (1982). Synthesis and degredation of heat shock proteins during development and decay of thermotolerance. *Cancer Research*. 42:2457-2461.

Larsen, P.L. (1993). Aging and resistance to oxidative damage in *Caenorhabditis elegans. Proceedings of National Academy of Sciences of the United States of America*. 90:8905-8909.

Larsen, S., Nielsen, J., Hansen, C.N., Nielsen, L.B., Wibrand, F., Stride, N., Daa Schroders, H., Boushel, R., Helge, J.W., Dela, F., Hey-Mogensen, M. (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *Journal of Physiology*. 590(14):3349-3360.

Li, G.C., Werb, Z. (1982). Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proceedings of the National Academy of Science, USA*. 70:3218-3222.

Li, J., Lee, A.S. (2006). Stress induction of GRP78/BiP and its role in cancer. *Current Molecular Medicine*. 6:45-54.

Li, S., Crenshaw III, B.E., Rawson, E.J., Simmons, D.M., Swanson, L.W., Rosenfeld, M.G. (1990). Dwarf locus mutants lacking three pituitary cell types results from mutations in the POU-domain gene pit-1. *Nature*. 347:528-533.

Li, Y., Huang, T.T., Carlson, E.J., Melov, S., Ursell, P.C., Olson, J.L., Noble, L.J., Yoshimura, M.P., Berger, C., Chan, P.H., Wallace, D.C., Epstein, C.J. (1995). Dialated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nature Genetics.* 11:376-381

Lin, Y.J., Seroude, L., Benzer, S. (1998). Extended lifespan and stress resistance in the *Drosophila* mutant *methuselah*. *Science.* 282:943-946. *Nature*. 443(19):787-795.

Lin, M.T., Flint Beal, M. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases.

Lithgow G.J., White, T.M., Hinerfeld, D.A., Johnson, T.E. (1994). Thermotolerance of a long-lived mutant of *Caenorhabditis elegans. Journal of Gerontology*.49:270-279

Lebovitz, R.M., Zhang, Z., Voge, H., Cartwright, J., Dionne, L., Lu, N., Huang, S., Matzuk, M.M. (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proceedings of the National Academy of Sciences of the USA.* 93:9782-9787

Madsen, M.A., Hsieh, C, Boylston, W.H., Flurkey, K., Harrison, D., Papaconstantinou, J. (2004). Altered oxidative stress response of the long-lived Snell dwarf mouse. *Biochemical and Biophysical Research Communications*. 318:998-1005.

Martindale, J.L., Holbrook, N.J. (2002). Cellular response to oxidative stress: signalling for suicide or survival. *Journal of Cellular Physiology*. 192:1-15.

Marzetti, E., Wohlgemuth, S.E., Lees, H.A., Chung, H., Giovannini, S., Leewenburgh, C. (2008). Age-related activation of mitochondrial caspase-independent apoptotic signalling in rat gastrocnemius muscle. *Mechanisms of Ageing and Development*. 129:542-549.

Marzetti, E., Hwang, J.C.Y., Lees, H.A., Wohlgemuth, S.E., Dupont-Versteegden, E.E., Carter, C.S., Bernabei, R., Leeuwenburgh, C. (2010). Mitochondrial death effectors: relevance to sarcopenia and disuse muscle atrophy. *Biochemica et Biophysica Acta*. 1800:235-244.

Maynard, S.P., Miller, R.A. (2006). Fibroblasts from long-lived Snell dwarf mice are resistant to oxygen-induced *in vitro* growth arrest. *Aging Cell*. 5:89-96.

McArdle, A., Dillmann, W.H., Mestril, R., Faulkner, J.A., Jackson, M.J. (2004). Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction. *Federation of American Societies for Experimental Biology Journal*. 18:355-357.55

McLean, P.J., Kawamata, H., Shariff, S., Hewtt, J., Sharma, N., Ueda, K., Breakefield, X.O., Hyman, B.T. (2002). TorsinA and heat shock proteins act as molecular chaperones: suppression of alphasynuclein aggregation *Journal of Neurochemistry.* 83:846-854.

McMillan, D.R., Xiao, X., Shao, L., Graves, K., Benjamin, I.J. (1998). Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *Journal of Biology and Chemistry*. 273:7523-7528.

Medawar, P.B. (1952). *An unsolved problem of biology. (Lewis, London).*

Meister, A., Anderson, M.E. (1983). Glutathione. *Annual Review of Biochemistry.* 52(1): 711-760.

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P.P., Lanfrancone, L., Pelicci, P.G. (1999). The p66Shc adaptor protein controls oxidative stress response and lifespan in mammals. *Nature*. 402:209-313.

Mignotte, B., Vayssiere, J.L. (1998). Mitochondria and apoptosis. *European Journal of Biochemistry.* 252:1-15

Milakovic, T., Johnson, G.V.W. (2006). Mitochondrial respiration and ATP production are significantly impaired in striatal cells expressing mutant Huntingtin. *Journal of Biological Chemistry*. 280:30773-30782.

Morrow, G., Kim, H.J., Pecheur, M., Kaul, S.C., Wadhwa, R., Tanguay, R.T. (2010). Protection from aging by small chaperones: A trade-off with cancer? *Annual New York Academy of Science*. 1197:65-75.

Moulais R, Meaume S, Raynaud-Simon A. (1999). Sarcopenia, hypermetabolism, and aging. *Journal of Biological Chemistry*. 280:30773-30782.

Muller, F.L., Lustgarten, M.S., Jang, Y., Richardson, A., Van Remmen, H. (2007). Trends in oxidative aging theories. *Free Radical Biology and Medicine.* 43:477-503.

Murakami, S., Johnson, T.E. (2006). A genetic pathway conferring life extension and resistance to UV stress in *Caenorhabditis elegans. Genetics*. 143(3):1207-1218

Murakami, S., Salomon, A., Miller, R.A. (2003). Multiplex stress resistance in cells in long-lived Dwarf mice. *Federation of American Societies for Experimental Biology Journal.* 17:1565-1566.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal.* 417:1-13.

Murshid, A., Eguchi, T., Calderwood, S.K. (2013). Stress proteins in aging and lifespan. *International Journal of Hyperthermia*. Early online:1-6.

Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E.K., Jones, P.K., Chanbari, H., Wataya, T., Shimohama, S., Chiba, S., Atwood, C.S., Petersen, R.B., Smith, M.A. (2001). Oxidative damage is the earliest event in Alzheimer disease. *Journal of Neuropathology and Experimental Neurology*. 60(8):759-767.

Ohashi. S.,Mori, A., Kurihara, N., Mitsumoto, Y., Nakai, M. (2006). Age-related severity of dopaminergic neurodegeneration to MPTP neurotoxicity causes motor dysfunction in C57BL/6 mice. *Neuroscience Letter*. 401:183-187.

Orzechowski, A., Grizard, J., Jank, M., Gajowska, B., Lokoxiejewska, M., Zaron-Teperek, M., Godlewskia, M. (2002). Dexamethasone-mediated regulation of death and differentiation of muscle cells. Is hydrogen peroxide involved in the process? *Reproduction Nutrition Development*. 42:197-216.

Ott, M, Robertson, J.D., Gogvadze, V., Zhivotovsky, B., Orrenius, S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. 1259-1263.

Ozawa, K., Miyazaki, M., Matsuhisa, M., Takano, K., Nakatani, Y., Hatazaki, M., Tamatani, T., Yamagata, K., Miyagawa, J., Kiatao, Y., Hori, O., Yamasaki, Y., Ogawa, S. (2005). The endoplasmic reticulum chaperone improves insulin resistance in Type 2 diabetes. *Diabetes*. 54(3):657-663.

Packer, M.A., Porteous, C.M., Murphy, M.P. (1996). Superoxide production by mitochondria in the presence of nitric oxide forms peroxynitrite. *International Union of Biochemistry and Molecular Biology Life.* 40(3):527-534.

Page, M.M., Richardson, J., Wiens, B.E., Tiedtke, E., Peters, C.W., Faure, P.A., Burnes, G., Stuart, J.A. (2010). Antioxidant enzyme activities are not broadly correlated with longevity in 14 vertebrate endotherm species. *Age*. 32:255-270.

Page, M.M., Stuart, J.A. (2012). Activities of DNA base excision repair enzymes in liver and brain correlate with body mass, but not lifespan. *Age*. 34:1195–1209.

Page, M.M., Withers, D.J., Selman, C. (2013). Longevity of insulin receptor substrate1 null mice is not associated with increased basal antioxidant protection or reduced oxidative damage. *Age*. 35(3):647-658.

Palacino, J.J., Sagi, D., Goldberg, M.S., Krauss, S., Motz, C., Wacker, M., Klose, J. (2004). Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *The Journal of Biological Chemistry*. 279:18614-18622.

Pearl, R. (1928). The rate of living. London: University of London Press.

Perez, V.I., Buffenstein, R., Masamsetti, V., Leonar, S., Salmon, A.B., Mele, J., Andziak, B., Yang, T., Edrey, Y, Friguet, B., Ward, W., Richarson, A., Chaudhuri, A. (2009a). Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole rat. *Proceedings of the National Academy of Sciences.* 106(9):3059-3064.

Perez, V.I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q., Ikeno, Y., Richardson, A. (2009b). Is the oxidative stress theory of aging dead? *Biochimica and Biophysica Acta*. 1790:1005-1014.

Petit, A., Kawarai, T., Paitel, E., Sanjo, N., Maj, M., Scheid, M., Chen, F., Gu, Y., Hasegawa, H., Salehi-Rad, S., Wang, L., Rogaeva, E., Fraser, P., Robinson, B., St George-Hyslop, P., Tandon, A. (2005). Wild-type Pink-1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. *Journal of Biological Chemistry*. 280:34025-34032.

Pesah, Y., Pham, T., Burgess, H., Middlebrooks, B., Verstreken, P., Zhou, Y., Harding, M., Bellen, H., Mardon, G. (2004). *Drosophila* parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. *Development*. 131:2183-2194.

Practico, D., Uryu, K., Leight, S., Trojanoswki, J.Q., Lee, V.M. (2001). Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer's disease. *Journal of Neuropathology and Experimental Neurology*. 21(12):4183-4187.

Radak, Z., Zhao, Z., Goto, S., Kaoitai, E. (2011). Age-associated neurodegeneration and oxidative damage to lipids, proteins, and DNA. *Molecular Aspects of Medicine.* 32:305-315.

Ran, Q., Kiang, H., Gu, M., Qi, W., Walert, C.A., Roberts, LJ 2nd, Herman, B., Richardson, A., Van Remmen, H. (2006). Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis. The *Journal of Biological Chemistry.* 279:55137-55146.

Reddy, P.H., McWeeney, S., Park, B.S., Manczak, M., Gutala, R.V., Partovi, D., Jung, Y., Yau, V., Searles, R., Mori, M, Quinn, J. (2004). Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and

apoptotic genes is an early cellular change in Alzheimer's disease. *Human Molecular Genetics*. 13(12):1225-1240.

Riabowol, K.T., Mizzen, L.A., Welch, W.J. (1988). Heat shock is lethal to fibroblasts microinjected with antibodies against HSP70. *Science*. 242(4877):433-436.

Robb, E., Page, M., Stuart, J. (2009). Mitochondria, cellular stress resistance, somatic cell depletion, and lifespan. *Current Ageing Science*. 2:12-27.

Robb, E.L., Maddalena, L.A, Dunlop, V.A., Foster, T., Stuart, J.A. (2012). Absence of metabolic rate allometry in an ex vivo model of mammalian skeletal muscle. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*. 162(3):157-162.

Robb, E.L., Christoff, C.A., Maddalena, L.A., Stuart, J.A. (2014). Mitochondrial reactive oxygen species in animal cells: relevance to aging and normal physiology. *Canadian Journal of Zoology*. 92:1-11.

Rojo, A.I., Montero, C., Salazar, M., Close, R.M., Fernandez-Ruiz, J., Sanchez-Gonzalez, M.A., de Sagarra, M.R., Jackson-Lewis, V., Cavada, C., Cuadrado, A. (2006). Persistent penetration of MPTP through the nasal route induces Parkinson's disease in mice. *European Journal of Neuroscience*. 24:1874-1884.

Rose, M.R. (1984). Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution.* 38(5):1004-1010.

Rose, M.R., Vu, L.N., Park, S.U., Graves, J.L. Jr. (1992). Selection on stress resistance increases longevity in *Drosophila melanogaster*. *Experimental Gerontology*. 27:241-250.

Rosenberg, I.H. (1997). Sarcopenia: origins and clinical relevance. *Journal of Nutrition*. 127:990S-1

Rosenblatt, J.S., Lunt, A.I., Parry, D.J., Partridge, T.A. (1995). Culturing satellite cells from living single muscle fibre explants. *In Vitro Cellular and Developmental Biology-Animal*. 31:773-779.

Salmon, A.B., Sadighi A.A., Buffenstein, R., Miller, R.A. (2008). Fibroblasts from naked mole-rats are resistant to multiple form of cell injury, but sensitive to peroxide, ultra-violet light, and endoplasmic reticulum stress. *Journal of Gerontology Series A: Biological Sciences and Medical Sciences*. 63:232-241.

Salmon, A.B., Richardson, A., Perez, V.I. (2009). Update on the oxidative stress theory of aging: Does oxidative stress play a role in aging or healthy aging? *Free Radical Biology and Medicine.* 48:642-655

Salway, K.D., Gallagher, E.J., Page, M.M., Stuart, J.A. (2011a). Higher levels of heat shock proteins in longer-lived mammals and birds. *Mechanism of Ageing and Development*. 132(6-7):287-297

Salway, K.D., Page, M.M., Faure, P.A., Burness, G., Stuart, J.A. (2011b). Enhanced protein repair and recycling are not correlated with longevity in 15 vertebrate endotherm species. *Age*. 33:33-47.

Samali, A., and Orrenius, S. (1998). Heat shock proteins, regulators of stress response and apoptosis. *Cell Stress & Chaperones*. 3(4):228-236.

Schapira, A. H. V., Cooper, J. M., Dexter, D., Clark, J. B., Jenner, P., Marsden, C. D. (1990). Mitochondrial complex I deficiency in Parkinson's disease. *Journal of Neurochemistry*. 54: 823–827.

Schapira, A.H.V. (2006). Mitochondrial disease. *Lancet*. 368:70-82.

Service, P.M., Hutchinson, E.W., Mackinley, M.D., Rose, M.R. (1985). Resistance to environmental stress in *Drosophila melanogaster* selected for postponed selection. *Physiological Zoology.* 58(4):380-389.

Sherman M. Y., Goldberg A. L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron*. 29:15–32.

Snell, G.D. (1929). Dward, a new Mendelian recessive character of the house mouse. *Proceedings of the National Academy of Science USA*. 15:733-734.

Sohal, R.S., Svensson, I., Sohal, B.H., Brunk, U.T. (1989). Superoxide anion radical production in different animal species. *Mechanisms of Ageing and Development*. 49:129-135.

Sohal, R.S., Sohal, B.H., Brunk, U.T. (1990a). Relationship between antioxidant defenses and longevity in different mammalian species. *Mechanisms of Ageing and Development.* 53:217-227.

Sohal, R.S., Svensson, E., Brunk, U.T. (1990b). Hydrogen peroxide production by liver mitochondria in different species. *Mechanisms of Ageing and Development*. 53:209-215.

Sohal, R.S., Ku, H.H., Agarwal, S. (1993). Biochemical correlates of longevity in two closely related rodent species. *Biochemical and Biophysical Research Community*. 196:7-11.

Song, W., Kwak, H.B., Lawler, J.M. (2006). Exercise training attenuates age-induced changes in apoptotic signalling in rat skeletal muscle. *Antioxidant Redox Signalling*. 8:517-528.

Spanier, B., Rubio-Aliaga, I., Hu, H., Daniel, H. (2010). Altered signalling from germline to intestine pushes daf-2;pept-1 *Caenorhabditis elegans* into extreme longevity. *Aging Cell*. 9(4):636-646.

Speakman, J.R. (2005). Body size, energy metabolism, and lifespan. *Journal of Experimental Biology*. 208:1717-1730.

Steele, A.D., Hutter, G., Jackson, W.S., Heppner, F.L., Borkowski, A.W., King, O.D., Raymond, G.J., Aguzzi, A., Lindquist, S. (2008). Heat shock factor 1 regulates lifespan as distinct from diseases onset in prion disease. *Proceedings of the National Academy of Science*. 105:13626-13631.

Stetler, R.A., Gan, Y., Zhang, W., Liou, A.K., Cao, Y., Cao, G., Chen, J. (2010). Heat shock proteins: cellular and molecular mechanisms in the central nervous system. *Progress in Neurobiology*. 92:184-211.

Stuart, J.A., Page, M.M. (2010). Plasma IGF-1 is negatively correlated with body mass in a comparison of 36 mammalian species. *Mechanisms of Ageing and Development*. 131:591–598

Subjeck, J.R., Sciandra, J.J., Johnson, R.J. (1982). *British Journal of Radiology*. 55:579-584.

Sun, J., Folk, D., Bradley, T.J., Tower, J. (2002). Induced overexpression of mitochondrial Mn-superoxide dismutase extends the lifespan of adult *Drosophila melanogaster. Genetics.* 161:661-672.

Swindell, W.R., Masternak, M.M., Kopchick, J.J., Conover, C.A., Bartke, A., Miller, R.A. (2009). Endocrine regulation of heat shock protein mRNA levels in long-lived dwarf mice. *Mechanisms of Ageing and Development*. 130:393-400.

Tedesco, F.S., Dellavalle, A., Diaz-Manera, J., Messina, G., Cossu, G. (2010). Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *Journal of Clinical Investigation*. 120:11-19.

Theil, L.E., Karin, M. (1993). Transcriptional control of GH expression and anterior pituitary development. *Endocrinology Review*. 14:670-689.

Tower, J. (2009). Hsps and aging. *Trends in Endocrinology and Metabolism*. 20(5):216-222.

Trinei M., Giorgio, M., Cicalese, A., Barozzi, S., Ventura, A., Migliaccio, E., Milia, E., Padura, I.M., Raker, V.A., Maccaarana M., Petronilli, V., Minucci, S., Bernardi, P., Lanfrancone L., Pelicci, P.G. (2002). A p53-p66Shc signaling pathway controls intracellular redox status, levels of oxidation-damaged DNA, and oxidative stress-induced apoptosis. *Oncogene*. 21(24):3872-3878.

Vanfleteren, J.R. (1993). Oxidative stress and aging in *Caenorhabditis elegans. Biochemical Journal.* 292:605-608.

Vanhooren, V., Liu, X., Desmyter, L., Fan, Y., Vanwalleghem, L., Molle, W.V., Dewaele, S., Praet, M., Contreras, R., Libert, C., Chen, C. (2008). Overexpression of heat shock protein 70 in mice is associated with growth retardation, tumor formation, and early death. *Rejuvenation Research*. 11(6): 1013-1020.

Van Remmen, H., Salvador, C., Yang, H., Huang, T.T., Epstein, C.J., Richardson, A. (1999). Characterization of the antioxidant status of the heterozygous manganese superoxide dismutase knockout mouse. *Archives of Biochemistry and Biophysics*. 363(1):91-97.

Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S.R., Alderson, N.L., Baynes, J.W., Epstein, C.J., Huang, T.T., Nelson, J., Strong, R., Richardson, A. (2003). A life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiological Genomics.* 16:29-37.

Veereshwarayya, V., Kuman, P., Rosen, K.M., Mestril, R., Querfurth, H.W. (2006). Differential effects of mitochondrial heat shock protein 60 and related molecular chaperones to prevent intracellular β-amyloid-induced inhibition of complex IV and limit apoptosis. *Journal of Biological Chemistry*. 281:29468-29478.

Wagner, M., Hermanns, I., Bittinger, F., Kirkpatrick, C.J. (1999). Induction of stress proteins in human endothelial cells by heavy metal ions and heat shock. *American Journal of Physiology*. 277:1026-1033.

Walker, G.A., Lithgow, G.J. (2003). Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin like signals. *Aging Cell*. 2:131-139.

Williams, F.C. (1957). Pleiotropy, natural selection, and the evolution of senescence. *Evolution*. *11*:398-411.

Winklhofer K. F., Tatzelt J., Haass C. (2008). The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *European Molecular Biology Organization Journal*. 27:336–349.

Wittstock, M., Rehfeldt, C., Nurnberg, G., Renne, U., Bruck, W., Mix, E., Zettl, U.K. (2003). Sesceptibility to apoptosis in difference murine muscle cell lines. *Journal of Muscle Research and Cell Motility*. 24:521-526.

Wyllie, A.H. (2010). "Where, O Death, Is Thy Sting?" A Brief Review of Apoptosis Biology. *Molecular Neurobiology*. 42:4-9.

Yamamoto, M., Clark, J.D., Pastor, J.V., Gurnani, P., Nandi, A., Kurosu, H., Miyoshi, M., Ogawa, Y., Castrillon, D.H., Rosenblatt, K.P., Kuro-o, M. (2005). Regulation of oxidative stress by the anti-aging hormone klotho. *Journal of Biological Chemistry*. 280:38029-38034.

Yang, Y., Gehrke, S., Imai, Y., Huang, Z., Ouvang, Y., Wang, J., Yang, L., Beal, M.F., Vogel, H., Lu, B. (2006). Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of *Drosophila* Pink-1 is rescued by Parkin. *Proceedings of the National Academy of Sciences of the United States of America*. 103(28):10793-10798.

Yokoyama, K., Fukumoto, K., Murakami, T., Harada, S., Hosono, R., Wadhwa, R., Mitsui, R., Ohkuma, S. (2002). Extended longevity of *Caenorhabditis elegans* by knowing in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75. *Federation of European Biochemical Societies*. 516:53-57.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. *Journal of Biology and Chemistry*. 273:33741-33749.

Zhang, J., Perry, G., Smith, M.A., Robertson, D., Olson, S.J., Graham, D.G., Montine, T.J. (1999). Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *The American Journal of Pathology*. 154(5):1423-1429.

Appendix I

Table 1. Non-transformed data for CS activity and GPx/GR ratio in isolated brain mitochondria from 9 different avian and mammalian species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM).

Species	MLSP	CS Activity (µmol/min/mg)	SEM	GPx/GR
Syrian hamster	3.9	1693	159.8	NA
Rat	4.5	3169	364.0	1.079
Quail	6.0	1064	183.7	9.624
Gerbil	6.3	1123	126.5	0.5601
Rabbit	9.0	602.6	23.07	0.5625
Guinea Pig (GP)	12.0	1333	202.8	2.142
Bat	19.0	452.9	25.20	0.8410
Goose	26.0	1539	224.6	6.426
Naked mole rat (NMR)	31.0	791.8	55.18	0.4554

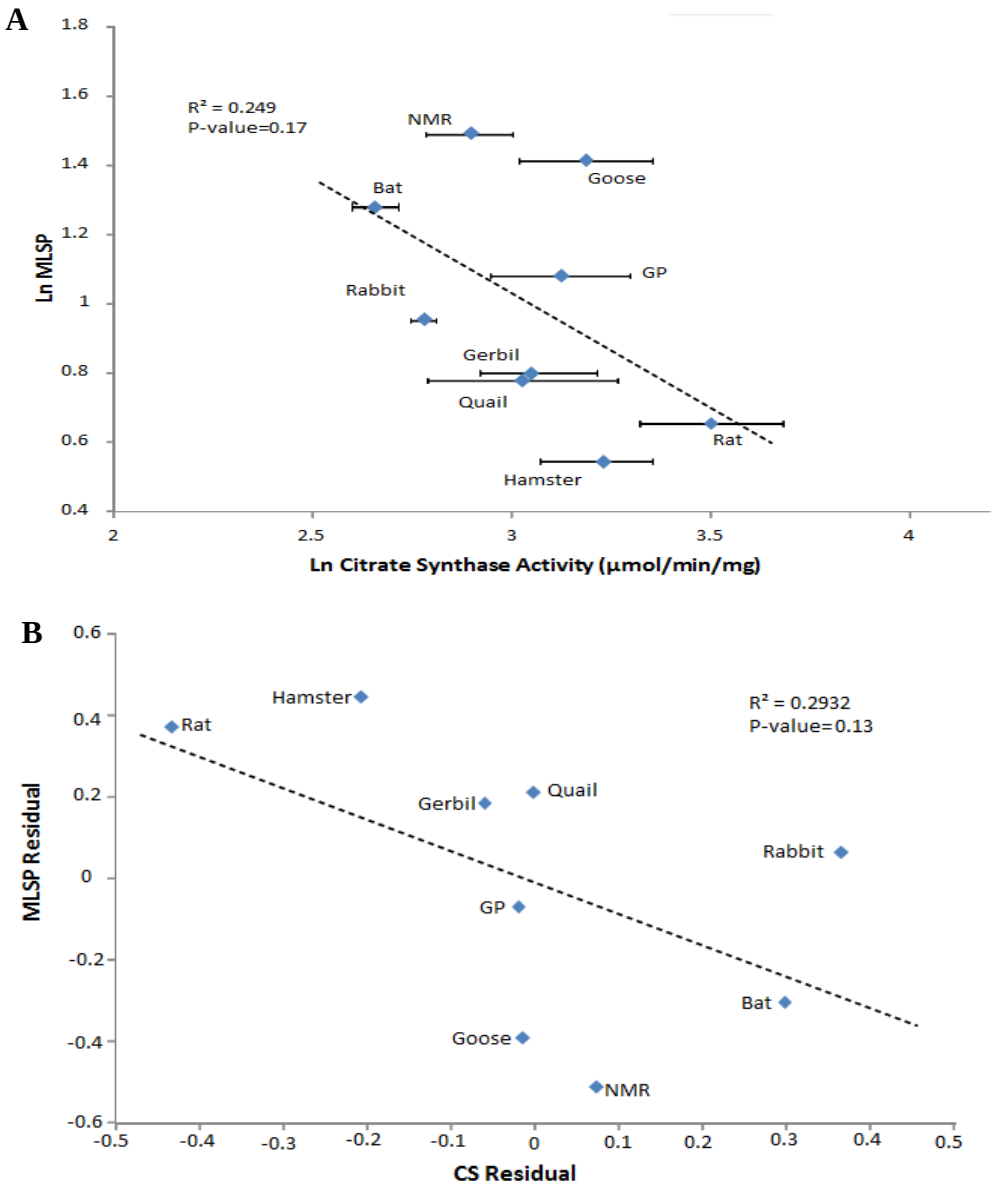

Figure 1: MLSP as a function of CS activity in isolated brain mitochondria from 9 endothermic species. A) MLSP is not correlated to CS activity. B) Residual analysis of MLSP is not correlated to CS activity. CS activity was measured in isolated brain mitochondria from nine endothermic vertebrate species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual).

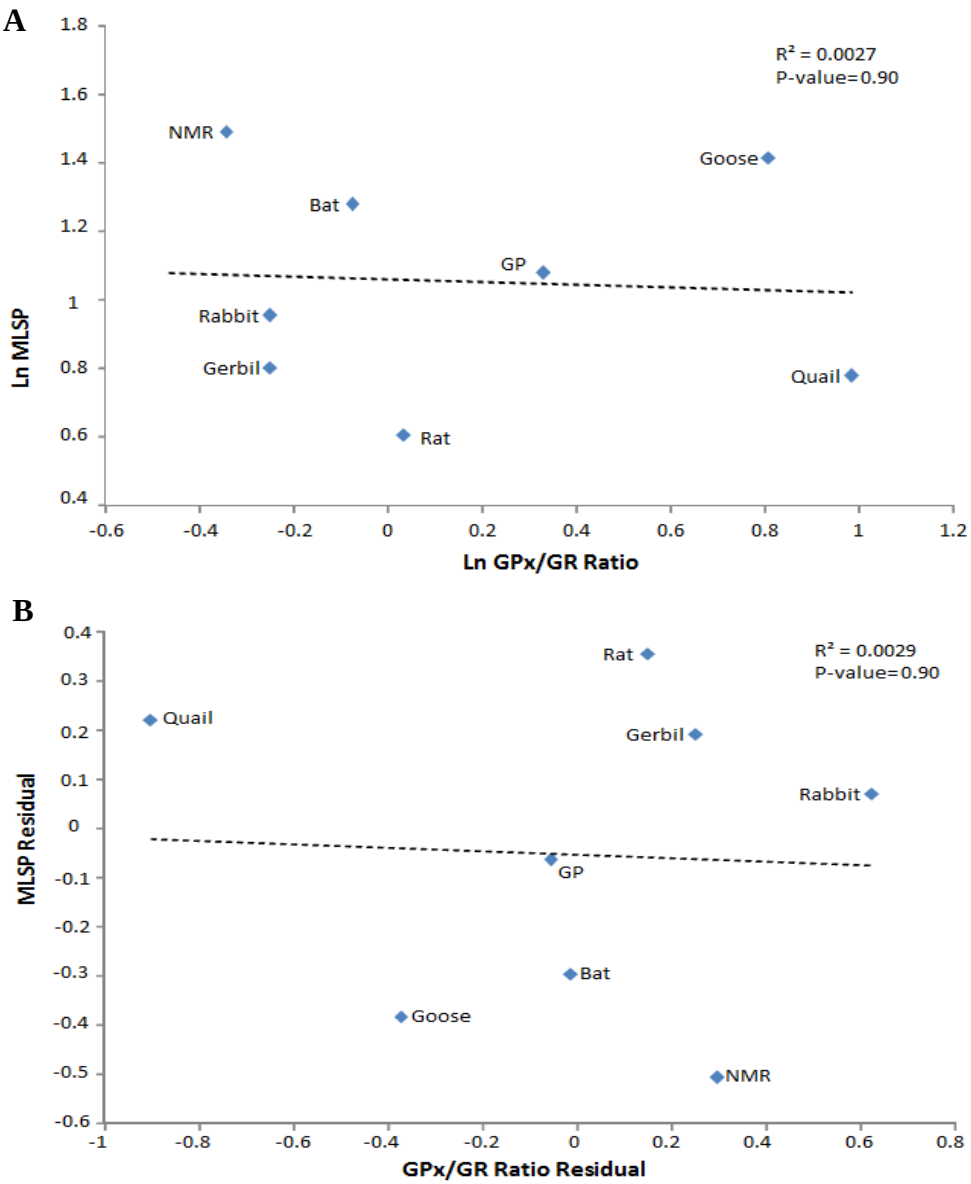

Figure 2: MLSP as a function of GPx/GR ratio in isolated brain mitochondria from 9 endothermic vertebrate species. A) MLSP is not correlated to GPx/GR ratio. B) Residual analysis of MLSP is not correlated to GPx/GR ratio. GPx/GR ratio was calculated by dividing the GPx activity (standardized to CS activity) by the GR activity (standardized to CS activity).

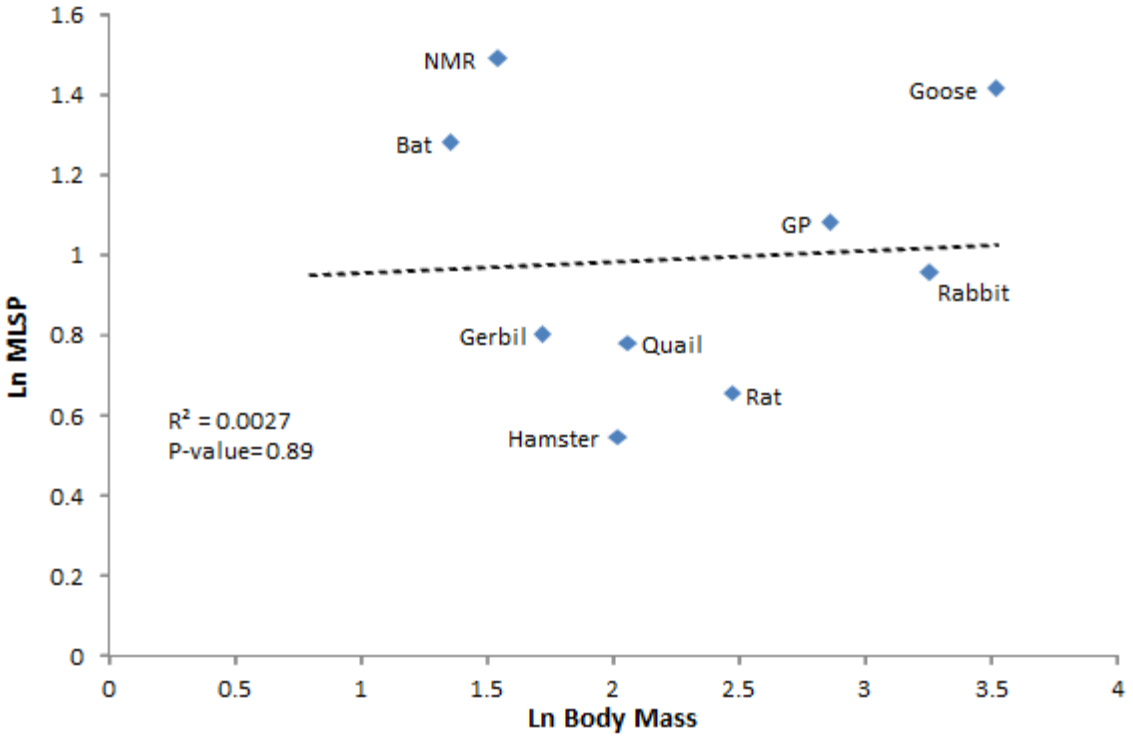

Figure 3: MLSP as a function of body mass for all mammalian and avian species used in this study. MLSP is not correlated with species body mass. Species weights and MLSP data are from AnAge (de Magalhaes et al., 2005). Residuals were calculated from this relationship and used in all other residual analysis.

MDDDIAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGD
EAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKAN
REKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHA
ILRLDLAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSS
SLEKSYELPDGQVITINERFRCPEALFQPSFLGMESCGIHETTFNSIMKCDVDIRKDLYAN
TVLSGGTTMYPGIARMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSFQQMWISK
QEYDESGPSIVHRKCF

Figure 4: Beta actin sequence for all mammalian species from which myoblasts were obtained. A sequence alignment using BLAST was performed which revealed that the sequence for beta actin is 100% homologous for all species used in Chapter 3.

Table 2. Non-transformed data for CS activity and beta actin levels in cultured mammalian myoblasts from 10 different species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM).

Species	MLSP	CS Activity	SEM	Beta Actin Levels	SEM
Syrian hamster	3.5	90.91	8.211	0.866	0.103
Mouse	4.5	233.8	24.05	0.408	0.0381
Rat	6.0	85.19	9.924	1.05	0.104
Gerbil	6.3	101.7	4.501	0.656	0.0388
Rabbit	9.0	157.2	21.59	1.14	0.153
Guinea pig (GP)	12.0	62.41	7.299	0.832	0.122
Cow	20.0	86.76	0.9965	1.07	0.117
Goat	20.8	88.46	7.152	0.790	0.151
Pig	27.0	67.02	6.836	0.955	0.126
Human	122.0	46.94	3.273	0.703	0.0456

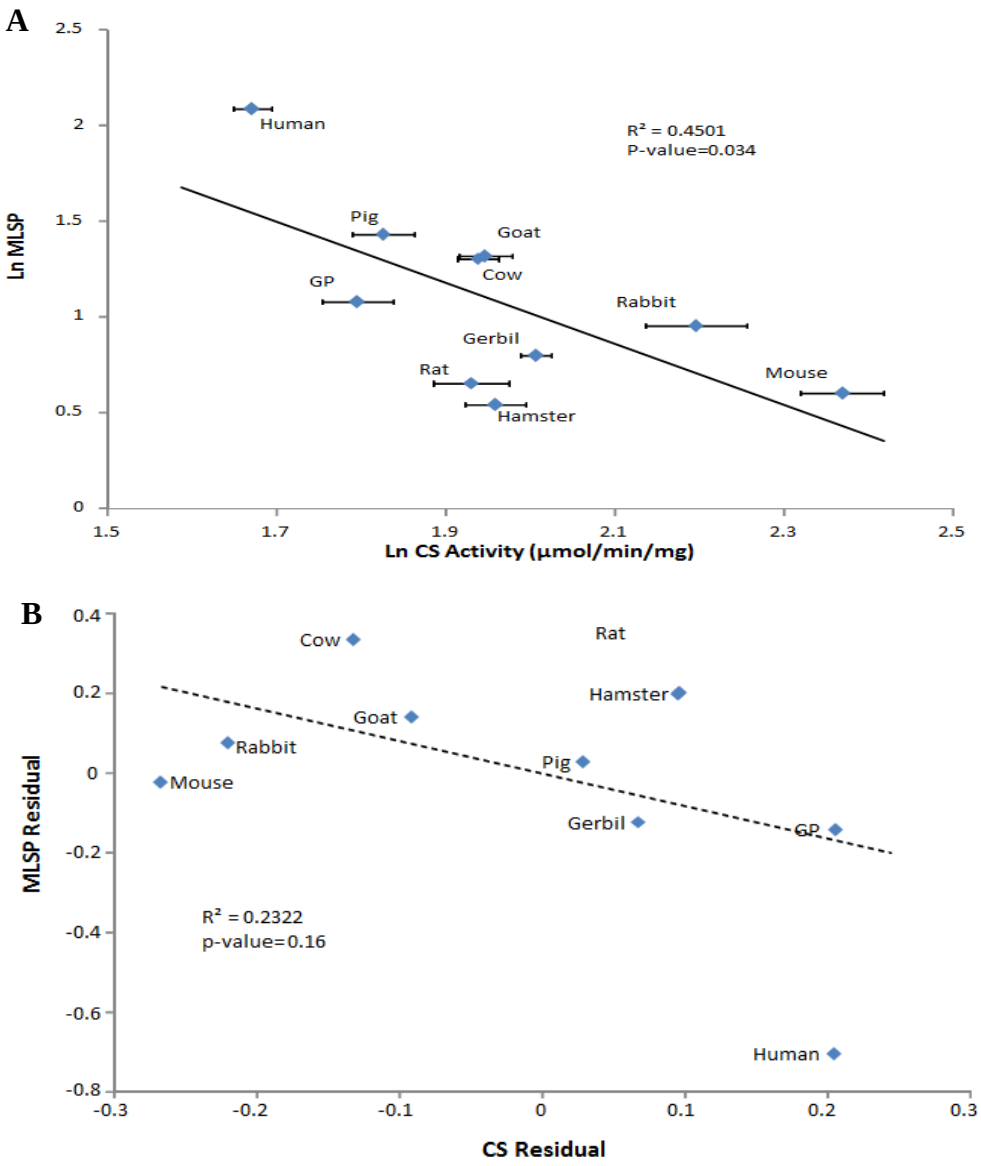

Figure 5: MLSP as a function of basal CS activity in cultured mammalian myoblasts. A) MLSP is negatively correlated to CS activity. B) Residual analysis of MLSP is not correlated to CS activity. CS activity was measured for 10 different species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual).

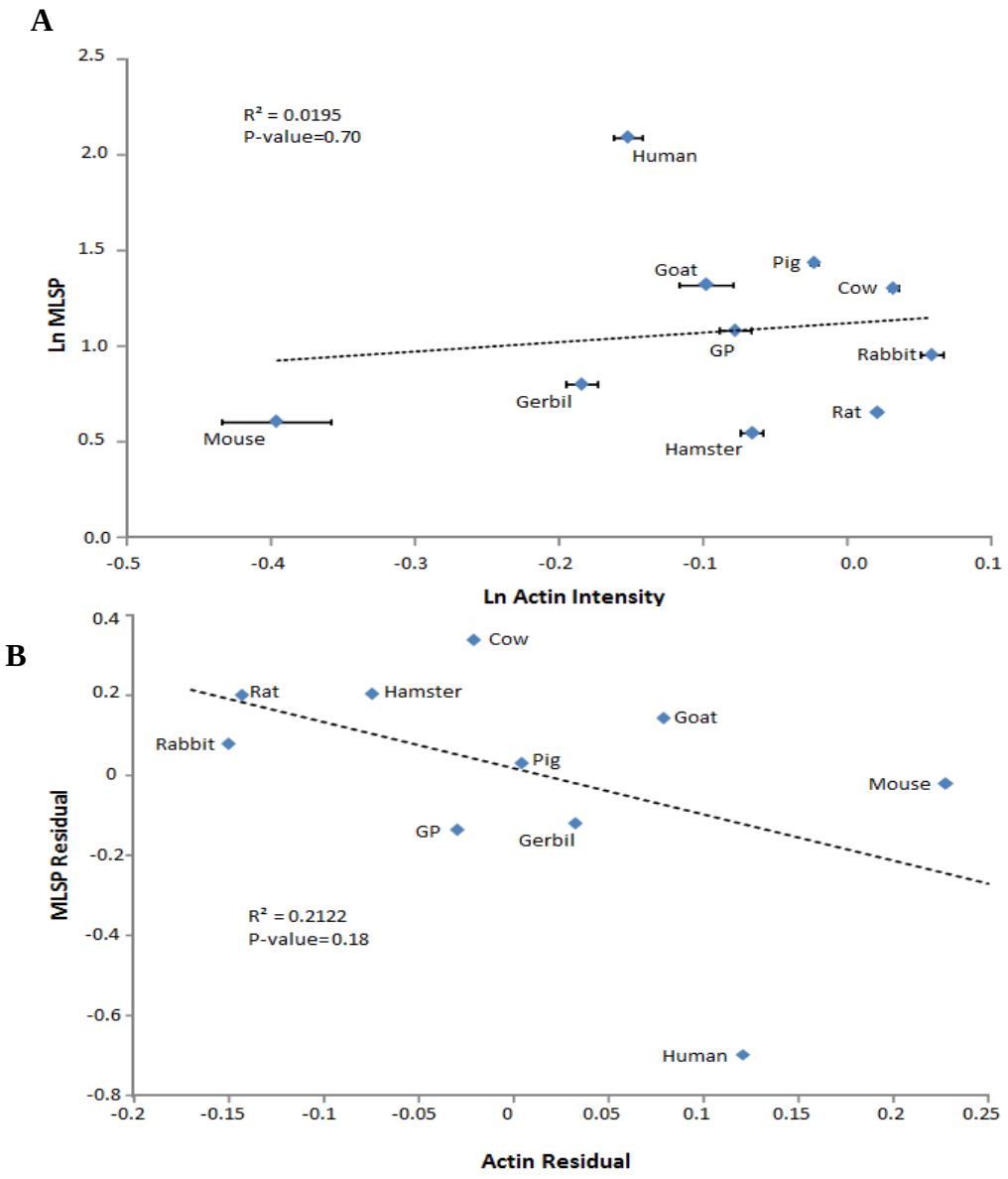

Figure 6: MLSP as a function of beta actin levels in cultured mammalian myoblasts.

A) MLSP is not correlated to beta actin levels. B) Residual analysis of MLSP is not correlated to beta actin levels. Beta actin levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual) and standardized to an internal control (guinea pig).

Table 3. Non-transformed data for HSP60 induction in cultured mammalian myoblasts from 10 different species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (St. Error). (GP=guinea pig).

Species	MLSP	HSP60 1 Hour	St. Error	HSP60 3 Hour	St. Error	HSP60 Post Recovery	St. Error
Mouse	4	0.8683	0.0453	0.9283	0.0395	1.1220	0.0722
Norway rat	4.5	1.3067	0.1054	0.9914	0.1000	1.2690	0.1717
Mongolian gerbil	6.3	1.0108	0.1117	0.9386	0.1142	1.1169	0.2729
Rabbit	9	1.0301	0.1548	1.1015	0.1261	1.2720	0.1472
Guinea pig	12	0.9407	0.1505	1.1069	0.0936	1.2611	0.0950
Cow	20	1.4291	0.1695	1.3543	0.0214	1.2708	0.0938
Goat	20.8	0.9599	0.0466	0.8954	0.0978	1.3076	0.0506
Pig	27	1.1221	0.1212	1.1660	0.0437	1.1314	0.1461
Human	122	1.7268	0.1844	1.5258	0.0970	1.4284	0.3242

Table 4. Non-transformed data for GRP78 induction in cultured mammalian myoblasts from 10 different species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (St. Error).

Species	MLSP	GRP78 1 Hour	St. Error	GRP78 3 Hour	St. Error	GRP78 Post Recovery	St. Error
Mouse	4	0.9213	0.058	0.8	0.0407	1.0469	0.1718
Norway rat	4.5	1.1683	0.0707	0.7965	0.1061	1.2798	0.0609
Mongolian gerbil	6.3	1.1357	0.1583	0.8913	0.082	1.4058	0.0566
Rabbit	9	1.0159	0.1177	1.0591	0.1736	1.0195	0.0894
Guinea pig	12	1.1387	0.1919	0.9568	0.0571	1.3311	0.0809
Cow	20	2.3809	0.4691	1.7418	0.1256	1.6318	0.2002
Goat	20.8	1.0151	0.0488	1.0807	0.0597	1.2676	0.0904
Pig	27	1.0707	0.1333	0.98	0.1064	1.1273	0.1462
Human	122	1.3013	0.1266	0.9786	0.1752	1.4049	0.1254

Table 5. Non-transformed data for CS induction following H_2O_2 exposure in cultured mammalian myoblasts from 10 different species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM).

Species	MLSP	CS 1 Hour	SEM	CS 3 Hour	SEM	CS Post Recovery	SEM
Mouse	4.0	1.23	0.149	0.835	0.124	1.15	0.193
Norway rat	4.5	1.39	0.133	0.833	0.101	1.07	0.0655
Mongolian gerbil	6.3	1.00	0.0301	0.862	0.0749	1.28	0.287
Rabbit	9.0	1.12	0.205	1.01	0.0890	1.06	0.113
Guinea pig (GP)	12.0	1.06	0.0445	0.879	0.0264	0.977	0.107
Cow	20.0	1.00	0.148	0.960	0.0171	1.10	0.114
Goat	20.8	0.913	0.102	1.04	0.129	0.931	0.0991
Pig	27.0	0.737	0.0318	1.15	0.191	1.06	0.107
Human	122.0	1.06	0.205	1.12	0.117	1.51	0.167

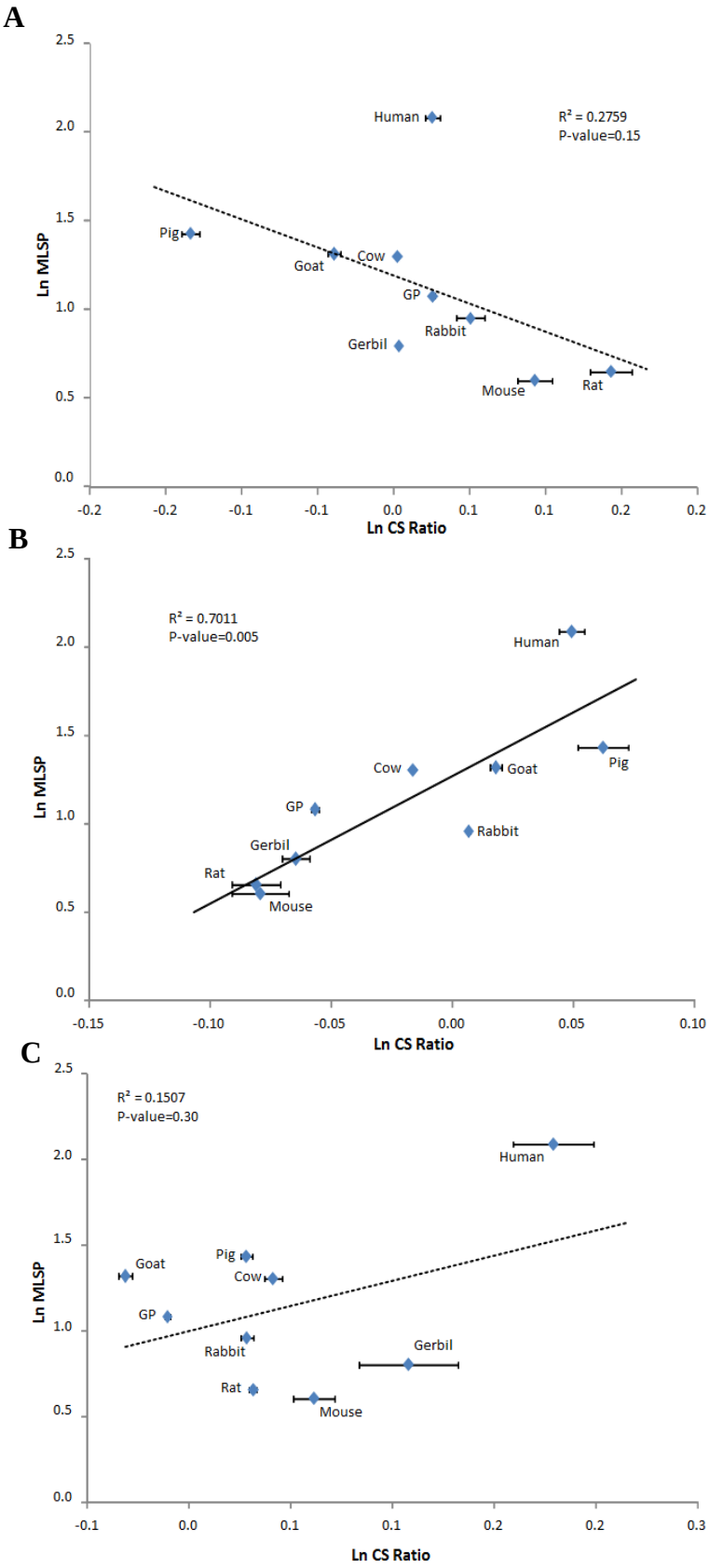

Figure 7: MLSP as a function of CS activity induction following H_2O_2 exposure. A) MLSP is not correlated CS activity induction after 1 hour H_2O_2 exposure. B) MLSP is positively correlated to CS activity induction after 3 hour H_2O_2 exposure. C) MLSP is positively correlated to CS activity induction after 3 hour recovery from H_2O_2 exposure. CS activities were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the CS activity levels from the H_2O_2 exposed cells by CS activity levels in the time matched unexposed cells

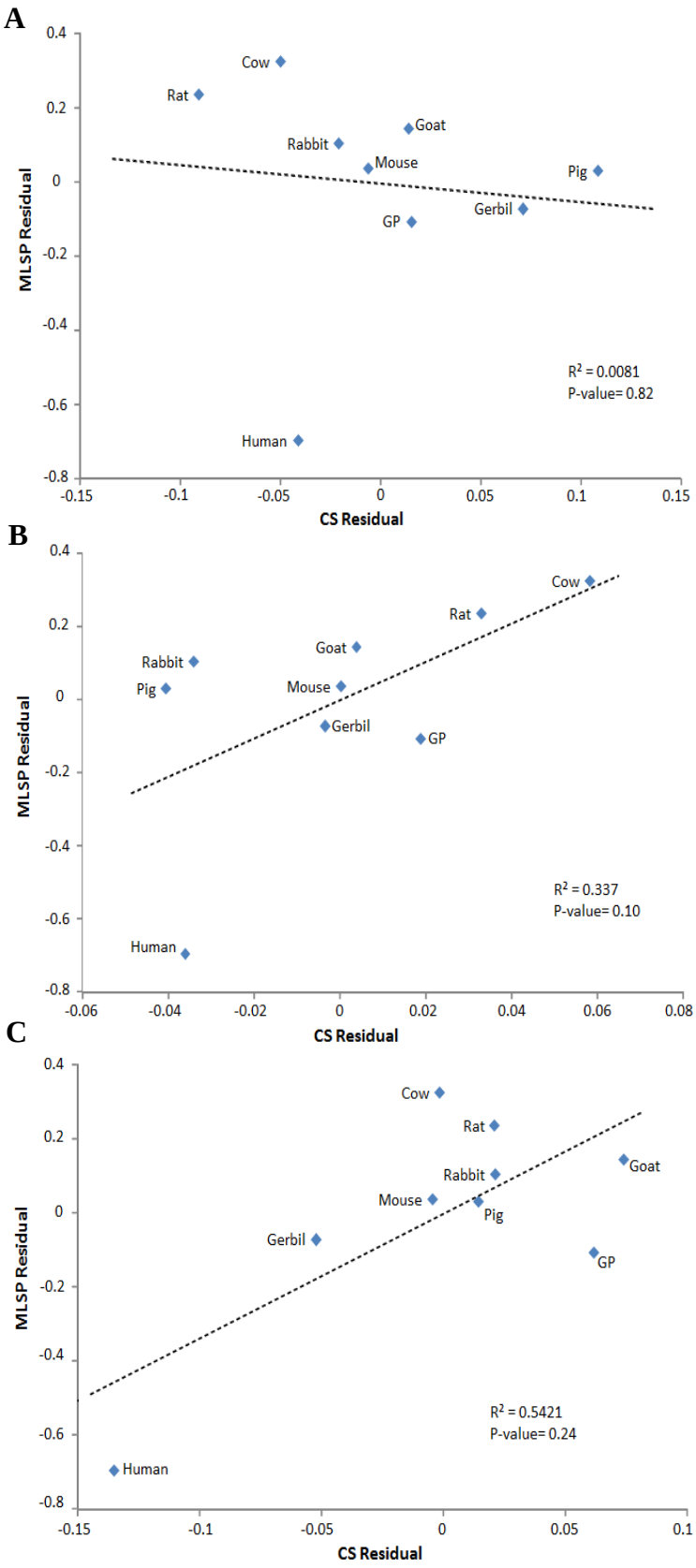

Figure 8: Residual analysis of MLSP as a function of CS activity induction following H_2O_2 exposure. A) MLSP is not correlated to CS activity induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to CS activity induction after 3 hour H_2O_2exposure in cultured mammalian myoblasts is not correlated to species MLSP. C) MLSP is not correlated to CS activity induction after 3 hour recovery from H_2O_2 exposure. CS activities were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the CS activity levels from the H_2O_2 exposed cells by CS activity levels in the time matched unexposed cells

Table 6. Non-transformed data for beta actin induction following H_2O_2 exposure in cultured mammalian myoblasts from 10 different species. Each value is an average of between 2-6 individuals of each species, and standard error of the mean is also shown (SEM). (GP=guinea pig).

Species	MLSP	Actin 1 Hour	SEM	Actin 3 Hour	SEM	Actin Post Recovery	SEM
Mouse	4.0	1.08	0.0444	1.15	0.201	1.11	0.102
Norway rat	4.5	1.08	0.112	1.07	0.155	1.46	0.216
Mongolian gerbil	6.3	1.18	0.190	0.894	0.121	1.50	0.389
Rabbit	9.0	0.981	0.179	1.17	0.247	1.39	0.230
Guinea pig (GP)	12.0	1.03	0.218	1.05	0.0643	1.09	0.101
Cow	20.0	1.27	0.199	0.927	0.0267	1.08	0.106
Goat	20.8	0.847	0.0472	1.02	0.0703	0.998	0.0586
Pig	27.0	0.764	0.162	0.884	0.0949	1.15	0.0583
Human	122.0	0.957	0.0157	0.766	0.0291	0.862	0.0258

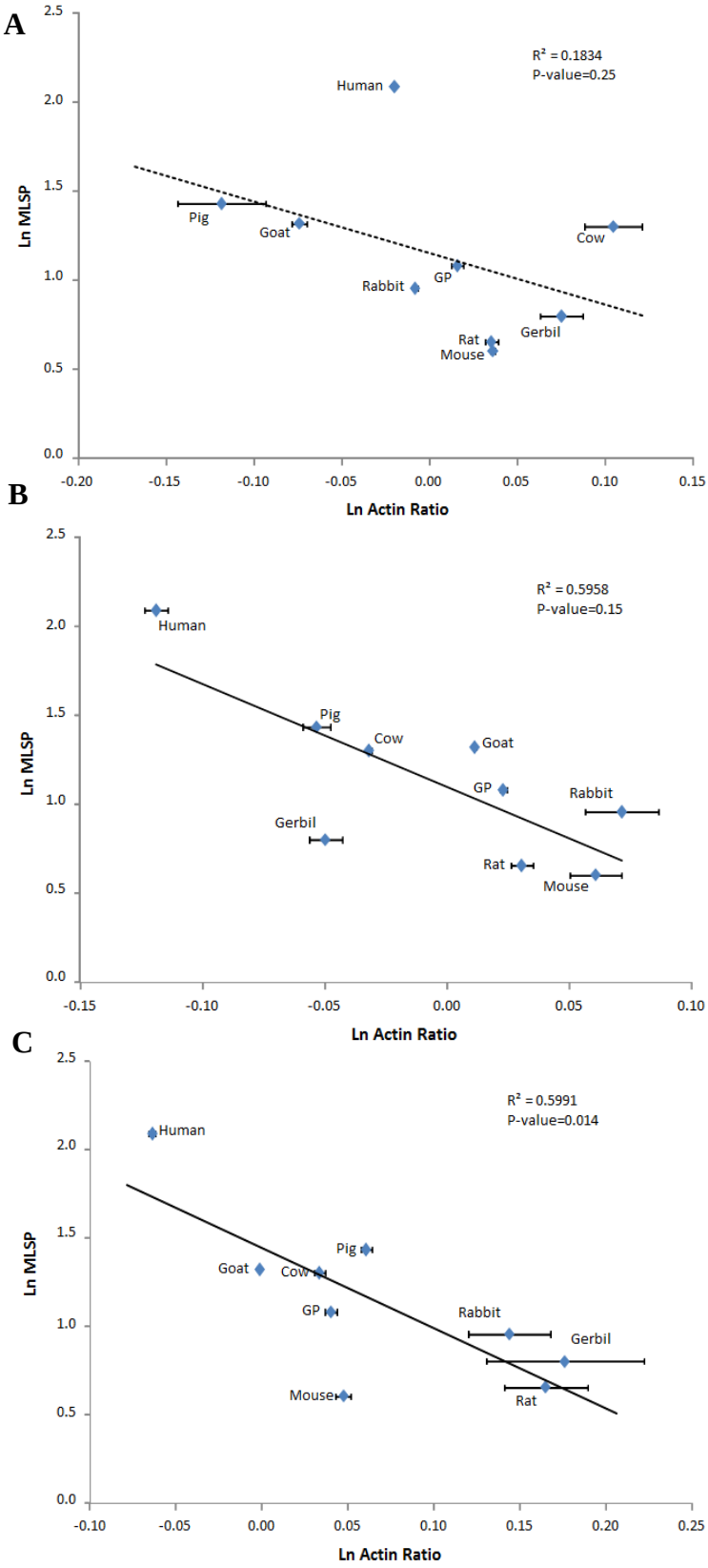

Figure 9: MLSP as a function of beta actin induction following H_2O_2 exposure. A) MLSP is not correlated to beta actin induction after 1 hour H_2O_2 exposure. B) MLSP is negatively correlated to beta actin induction after 3 hour H_2O_2 exposure.
C) MLSP is negatively correlated to beta actin induction after 3 hour recovery from H_2O_2 exposure. Beta actin levels were measured in cultured myoblasts from 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the beta actin levels from the stressed cells by beta actin levels in the time matched control cells.

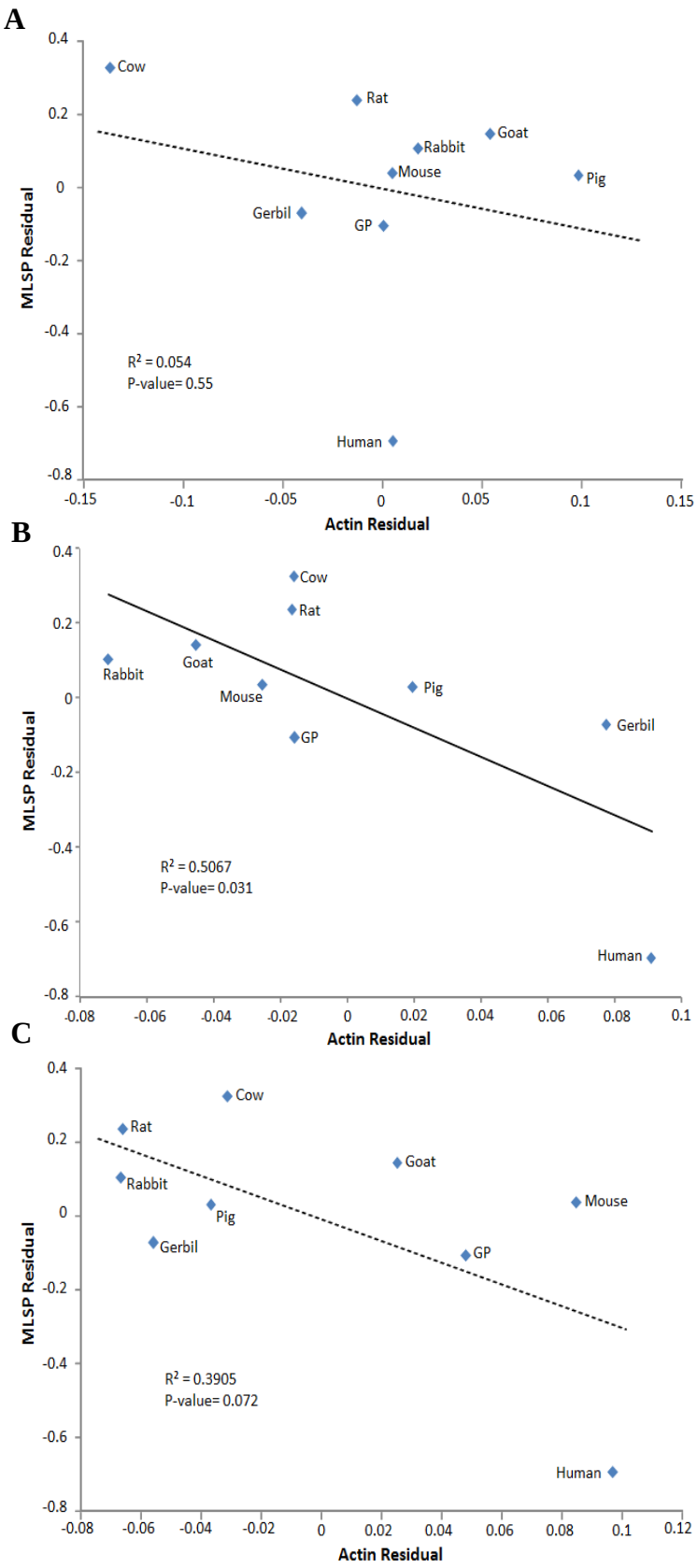

Figure 10: Residual analysis of MLSP as a function of beta actin induction following H_2O_2 exposure. A) MLSP is not correlated to beta actin induction after 1 hour H_2O_2 exposure. B) MLSP is negatively correlated to beta actin induction after 3 hour H_2O_2 exposure.
C) MLSP is not correlated to beta actin induction after 3 hour recovery from H_2O_2 exposure. Beta actin levels were measured in cultured myoblasts from 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the beta actin levels from the stressed cells by beta actin levels in the time matched control cells.

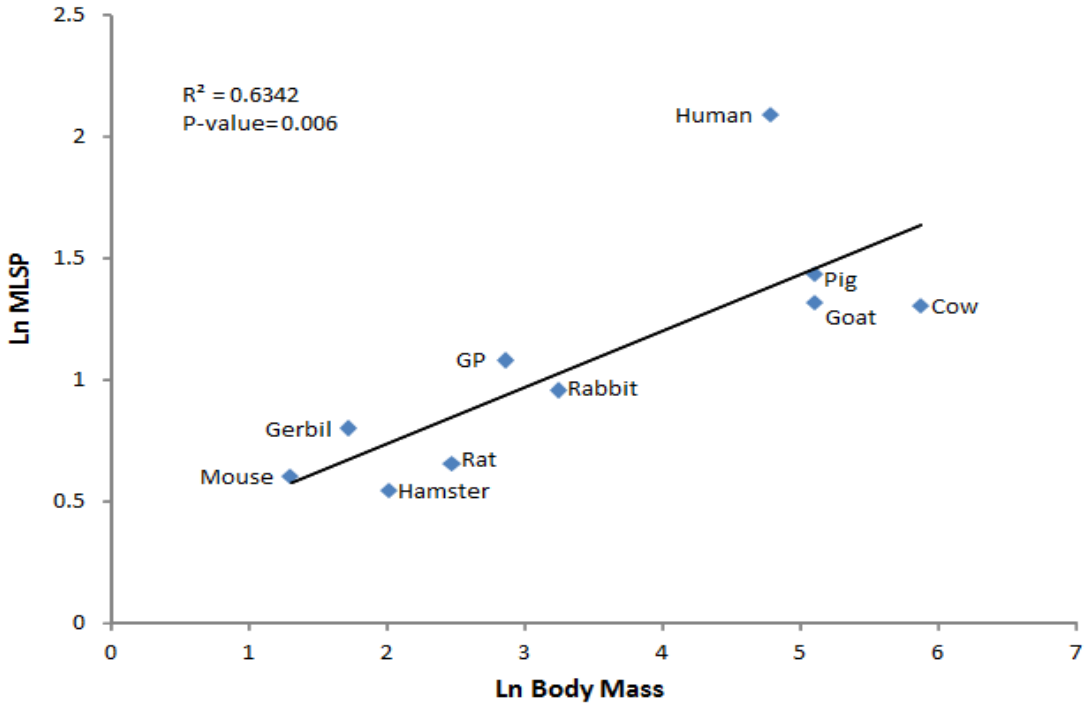

Figure 11: MLSP as a function of body mass for all mammalian species used in this study. MLSP is strongly correlated with mammalian body mass. Species weights and MLSP data are from AnAge (de Magalhaes et al., 2005). Residuals were calculated from this relationship and used in all other residual analysis.

Appendix II: Human MLSP of 90 years

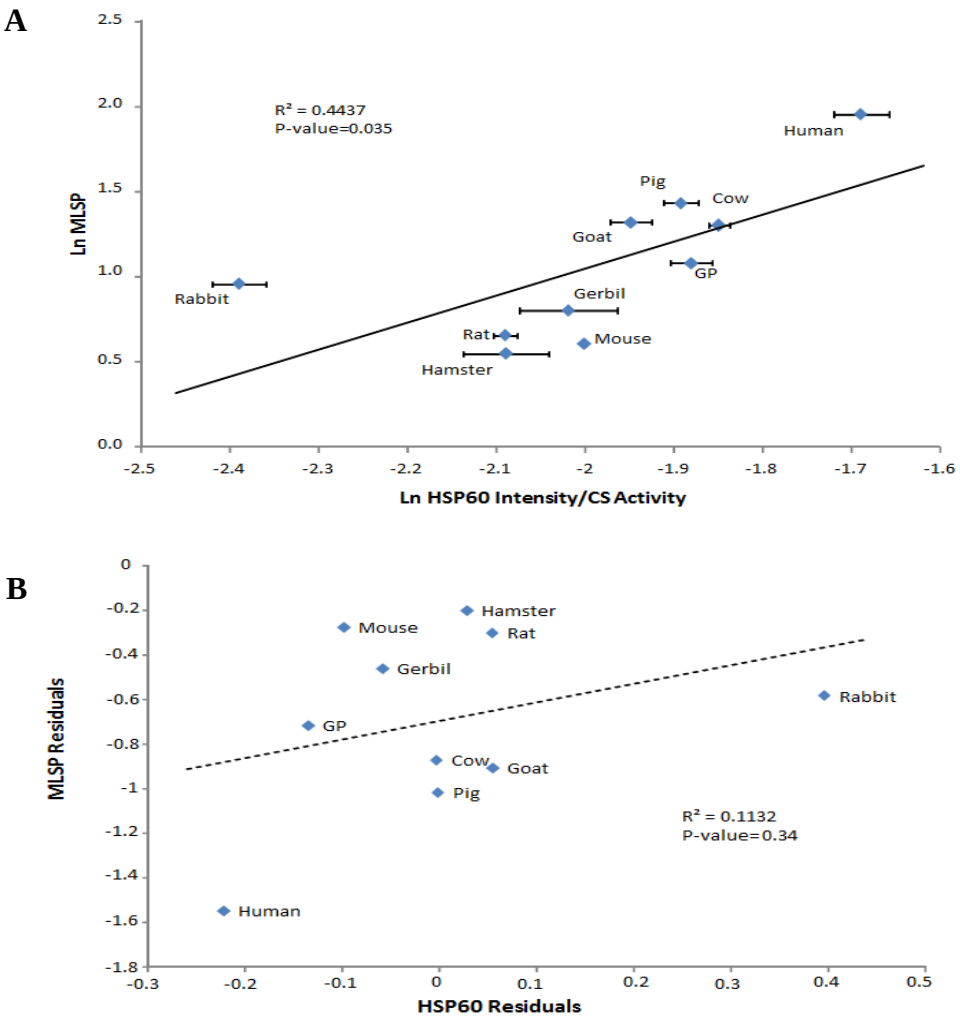

Figure 1: MLSP as a function of HSP60 levels in cultured mammalian myoblasts after standardization to beta actin and CS activity. A) MLSP is positively correlated to HSP60 levels. B) Residual analysis MSLP is not correlated to HSP60 levels. HSP60 levels were measured and standardized to the species aerobic capacity (measured as CS activity (µmol/min/mg)) for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual) and standardized to an internal control (guinea pig).

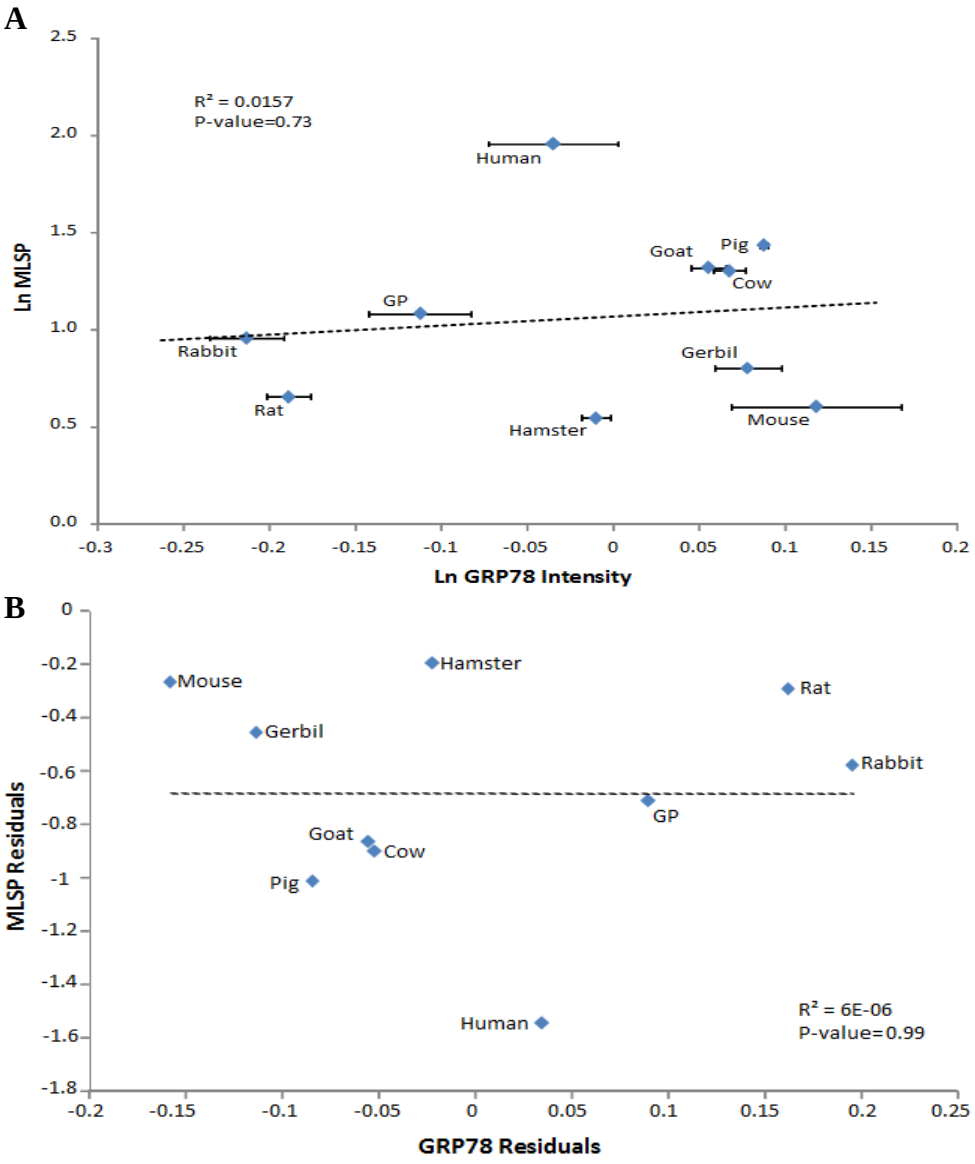

Figure 2: MLSP as a function of GRP78 levels in cultured mammalian myoblasts after standardization to beta actin. A) MLSP is not correlated to GRP78 levels. B) Residual analysis of MLSP is not correlated to GRP78. GRP78 levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual) and standardized to an internal control (guinea pig).

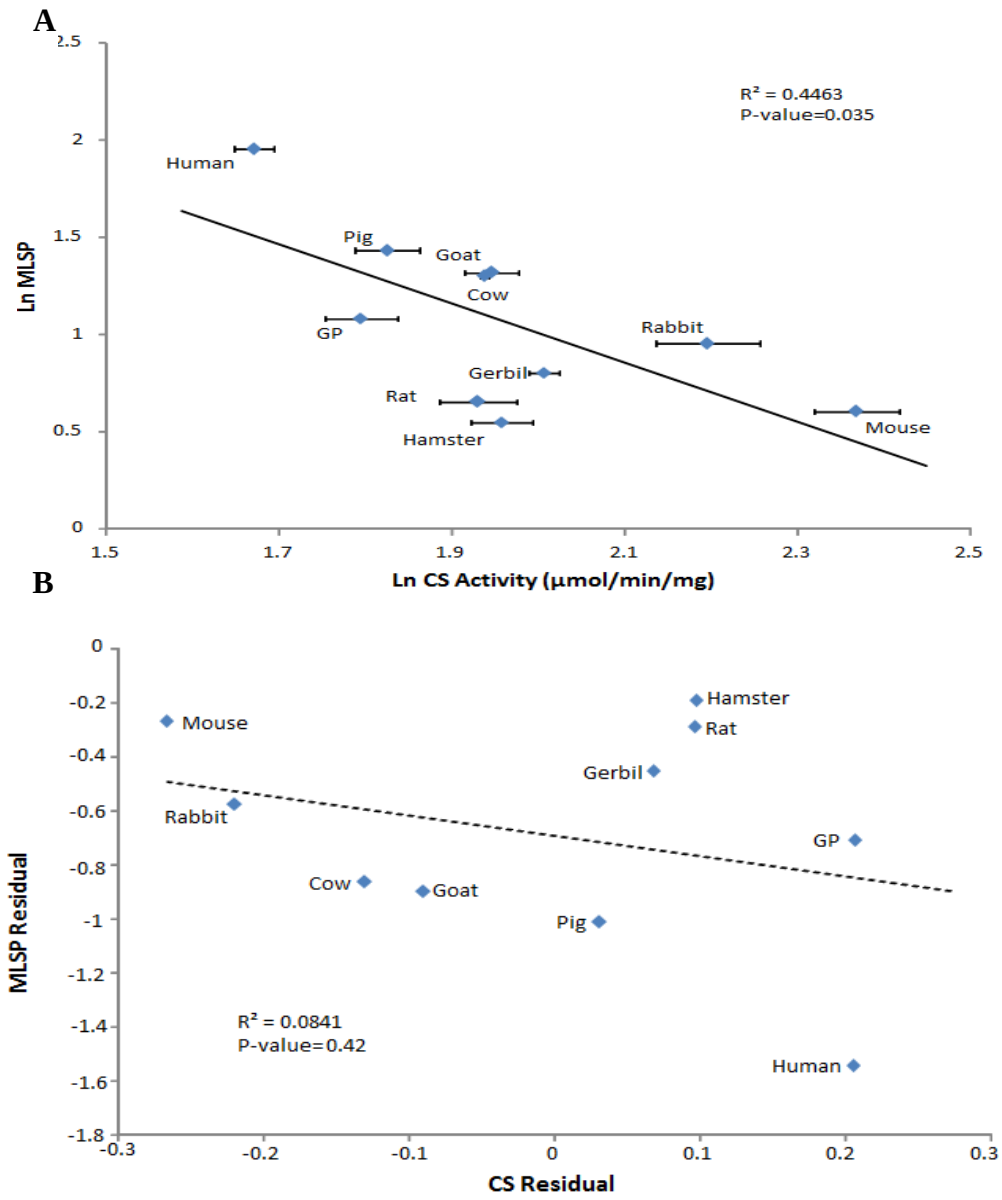

Figure 3: MLSP as a function of basal CS activity in cultured mammalian myoblasts. A) MLSP is negatively correlated to CS activity. B) Residual analysis of MLSP is not correlated to CS activity. CS activity was measured for 10 different species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual).

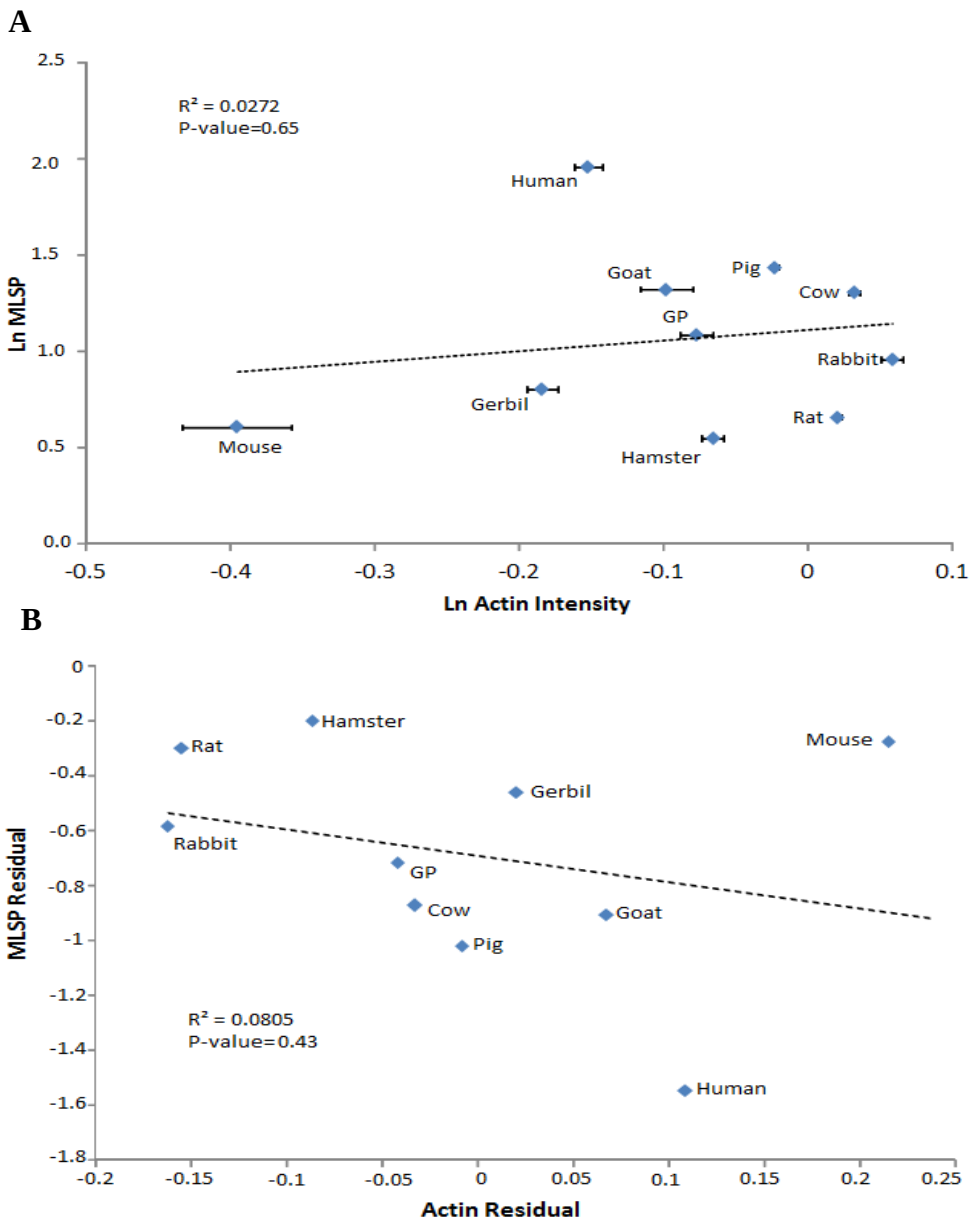

Figure 4: MLSP as a function of beta actin levels in cultured mammalian myoblasts.

A) MLSP is not correlated to beta actin levels. B) Residual analysis of MLSP is not correlated to beta actin levels. Beta actin levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual) and standardized to an internal control (guinea pig).

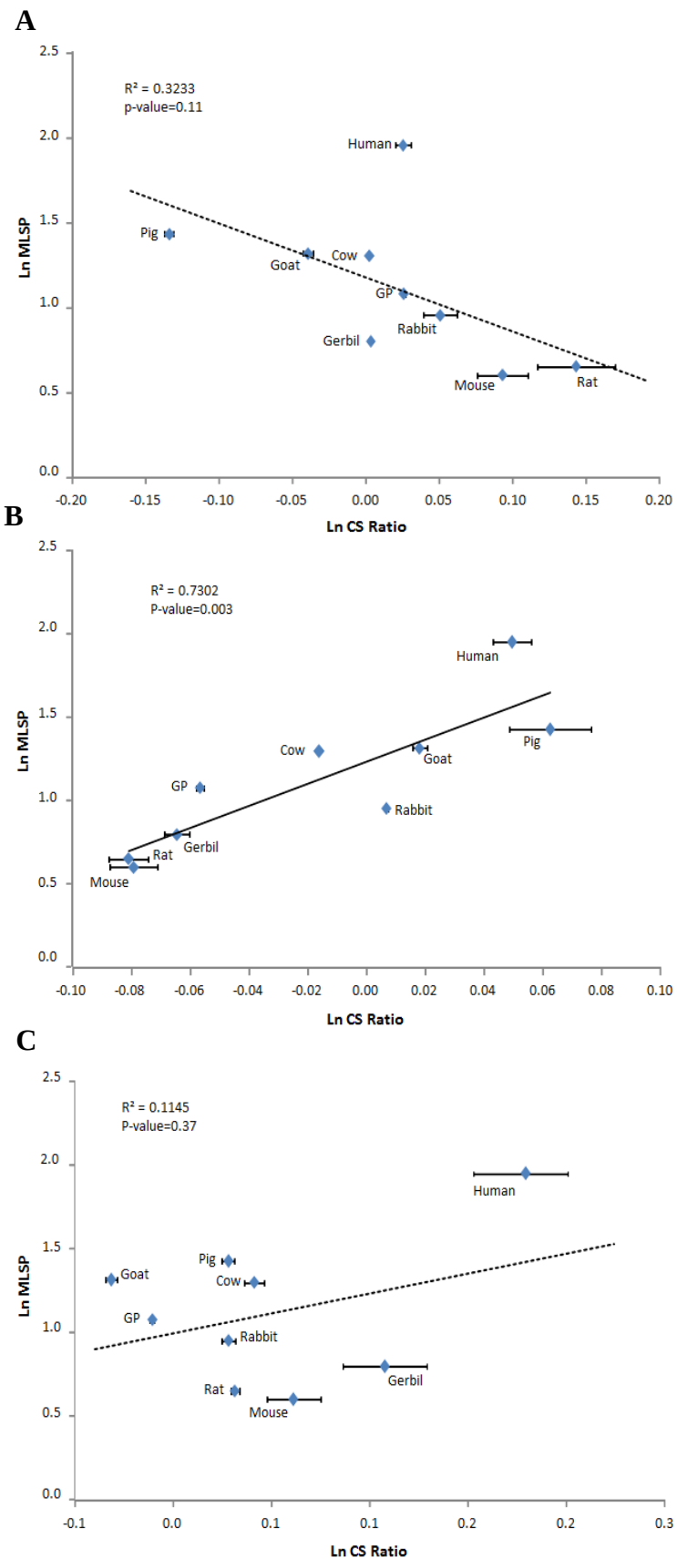

Figure 5: MLSP as a function of CS activity induction following H_2O_2 exposure. A) MLSP is not correlated CS activity induction after 1 hour H_2O_2 exposure. B) MLSP is positively correlated to CS activity induction after 3 hour H_2O_2 exposure. C) MLSP is positively correlated to CS activity induction after 3 hour recovery from H_2O_2 exposure. CS activities were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the CS activity levels from the H_2O_2 exposed cells by CS activity levels in the time matched unexposed cells

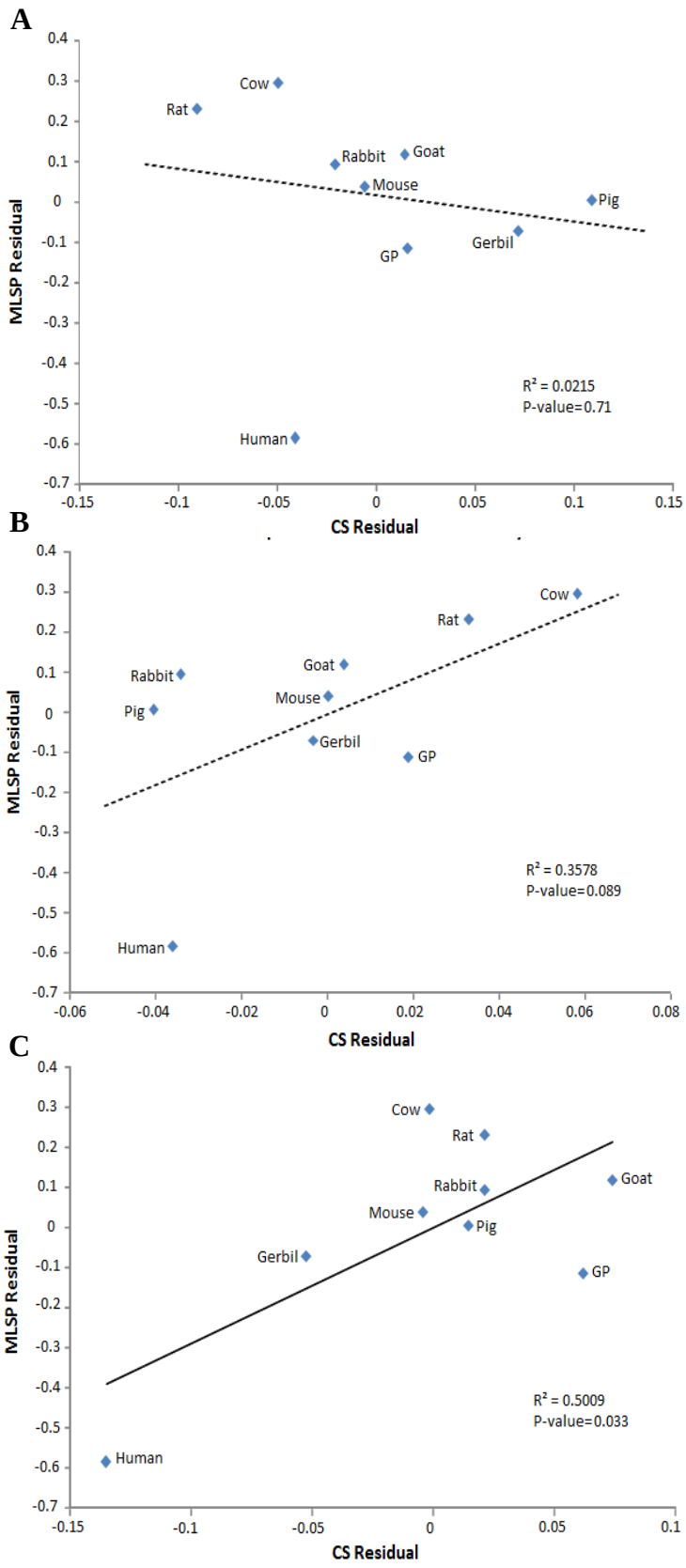

Figure 6: Residual analysis of MLSP as a function of CS activity induction following H_2O_2 exposure. A) MLSP is not correlated to CS activity induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to CS activity induction after 3 hour H_2O_2exposure in cultured mammalian myoblasts is not correlated to species MLSP. C) MLSP is not correlated to CS activity induction after 3 hour recovery from H_2O_2 exposure. CS activities were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the CS activity levels from the H_2O_2 exposed cells by CS activity levels in the time matched unexposed cells

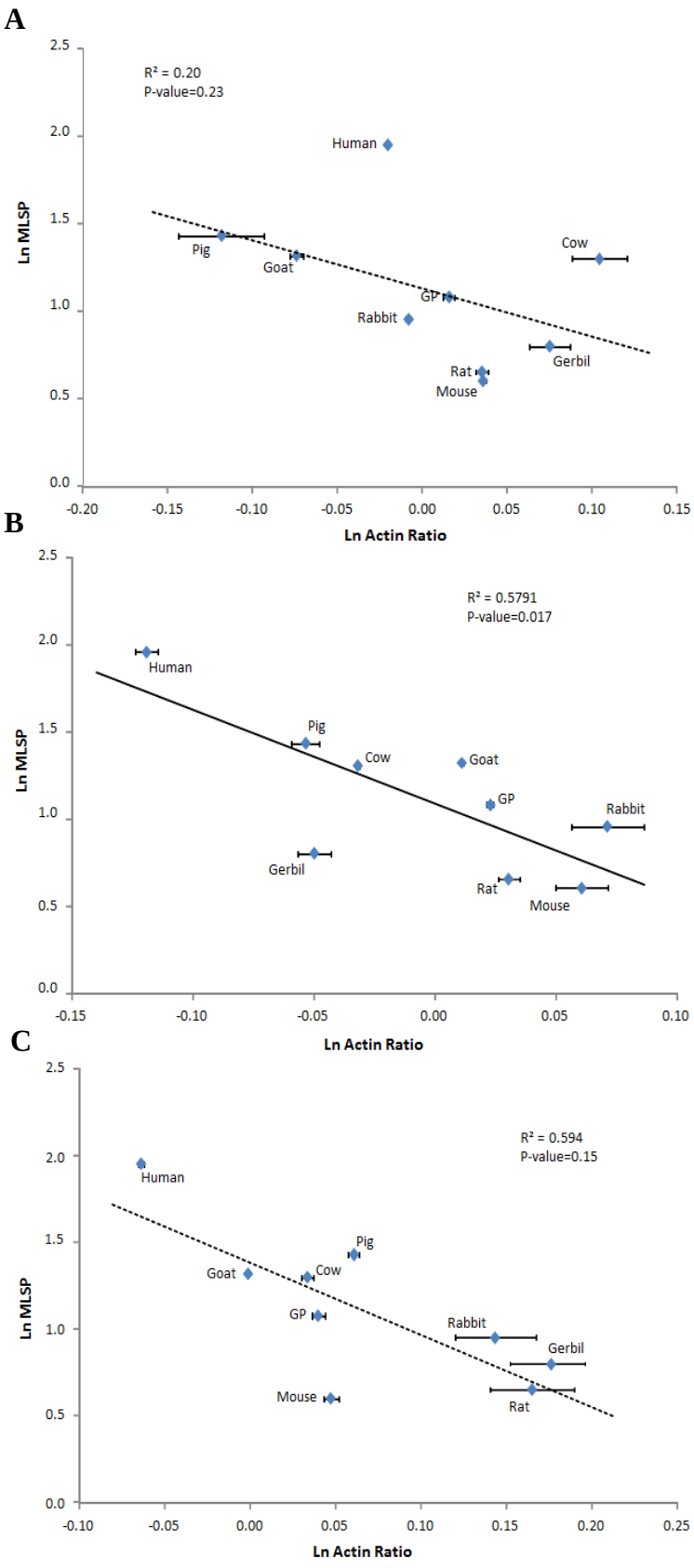

Figure 7: MLSP as a function of beta actin induction following H_2O_2 exposure. A) MLSP is not correlated to beta actin induction after 1 hour H_2O_2 exposure. B) MLSP is negatively correlated to beta actin induction after 3 hour H_2O_2 exposure. C) MLSP is negatively correlated to beta actin induction after 3 hour recovery from H_2O_2 exposure. Beta actin levels were measured in cultured myoblasts from 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the beta actin levels from the stressed cells by beta actin levels in the time matched control cells.

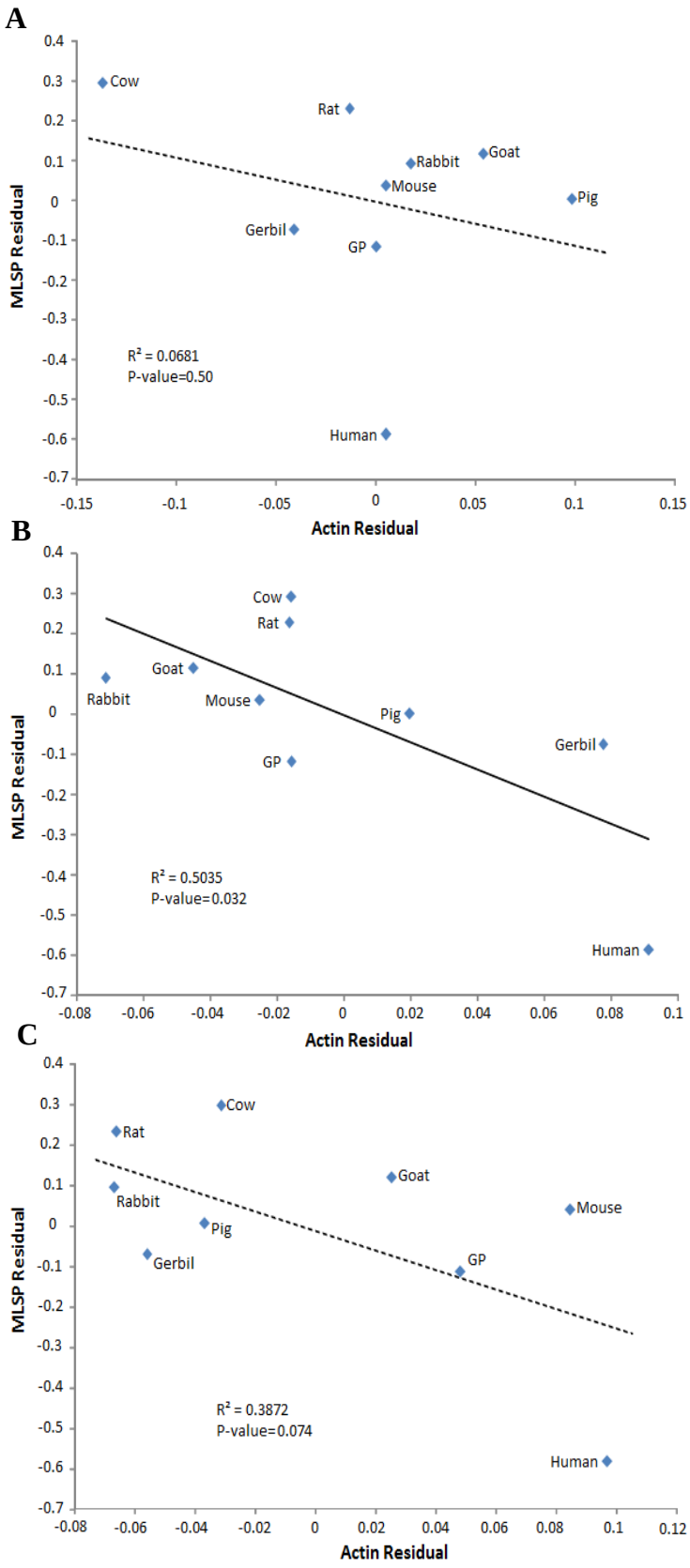

Figure 8: Residual analysis of MLSP as a function of beta actin induction following H_2O_2 exposure A) MLSP is not correlated to beta actin induction after 1 hour H_2O_2 exposure. B) MLSP is negatively correlated to beta actin induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to beta actin induction after 3 hour recovery from H_2O_2 exposure. Beta actin levels were measured in cultured myoblasts from 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the beta actin levels from the stressed cells by beta actin levels in the time matched control cells.

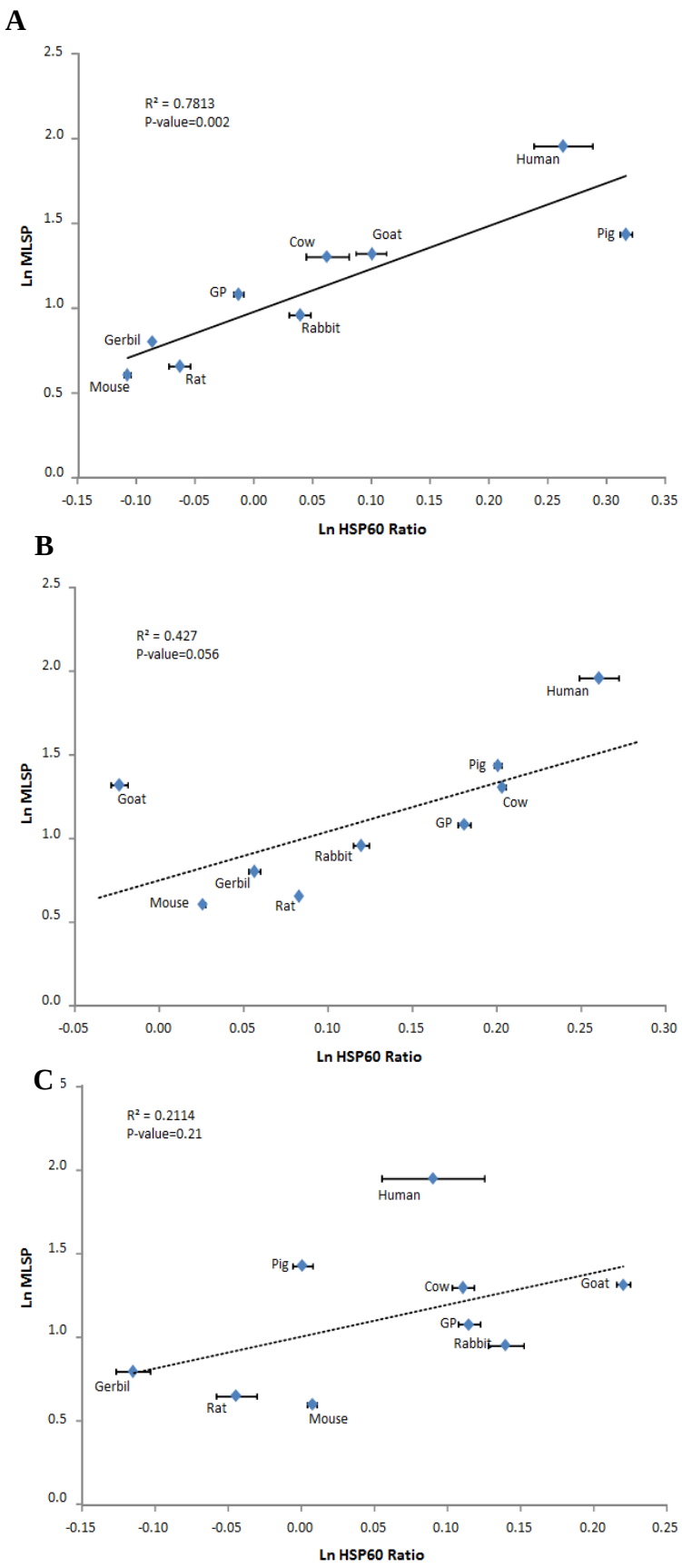

Figure 9: MLSP as a function of HSP60 induction following H_2O_2 exposure after standardization to beta actin and aerobic capacity. A) MLSP is positively correlated to HSP60 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to HSP60 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to HSP60 induction after 3 hour recovery from 3 hour H_2O_2 exposure. HSP60 levels were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the HSP60 levels from the H_2O_2 exposed cells by HSP60 levels in the time matched unexposed cells. Mitochondrial abundance was measured as CS activity (µmol/min/mg).

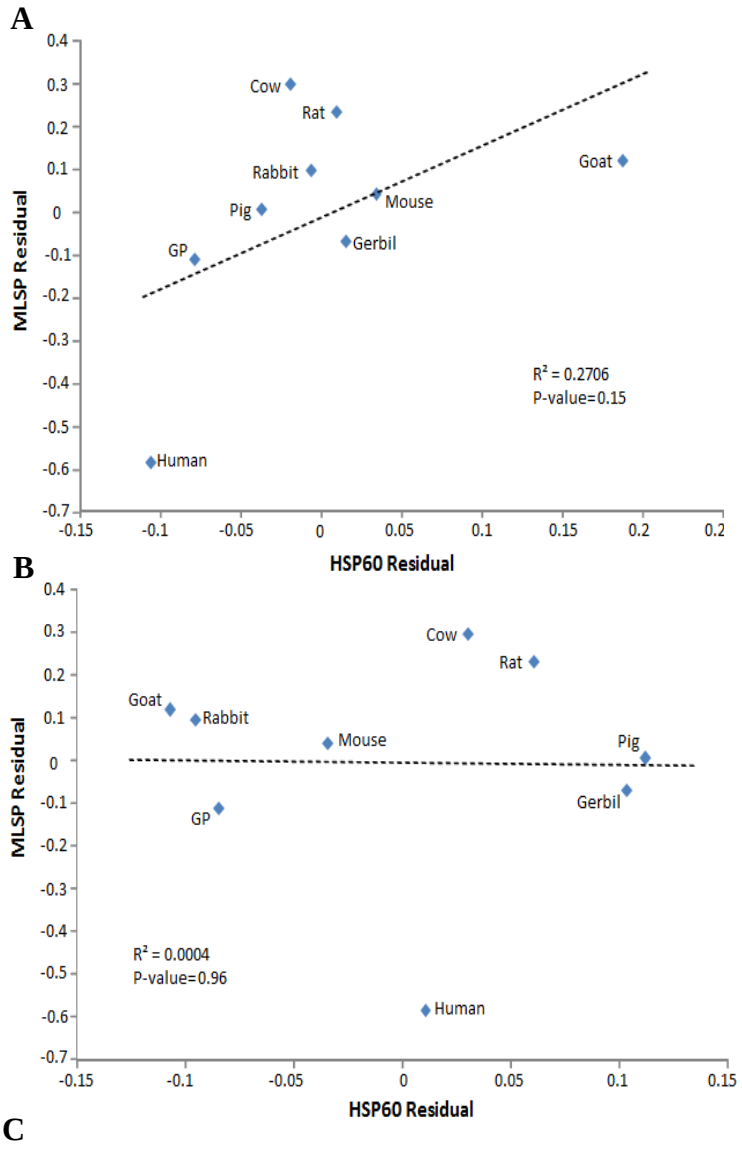

Figure 10: Residual analysis of MLSP as a function of HSP60 induction following H_2O_2 exposure after standardization to beta actin and aerobic capacity. A) MLSP is correlated to HSP60 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to HSP60 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to HSP60 induction after 3 hour recovery from 3 hour H_2O_2 exposure. HSP60 levels were measured for 9 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the HSP60 levels from the H_2O_2 exposed cells by HSP60 levels in the time matched unexposed cells. Mitochondrial abundance was measured as CS activity (µmol/min/mg).

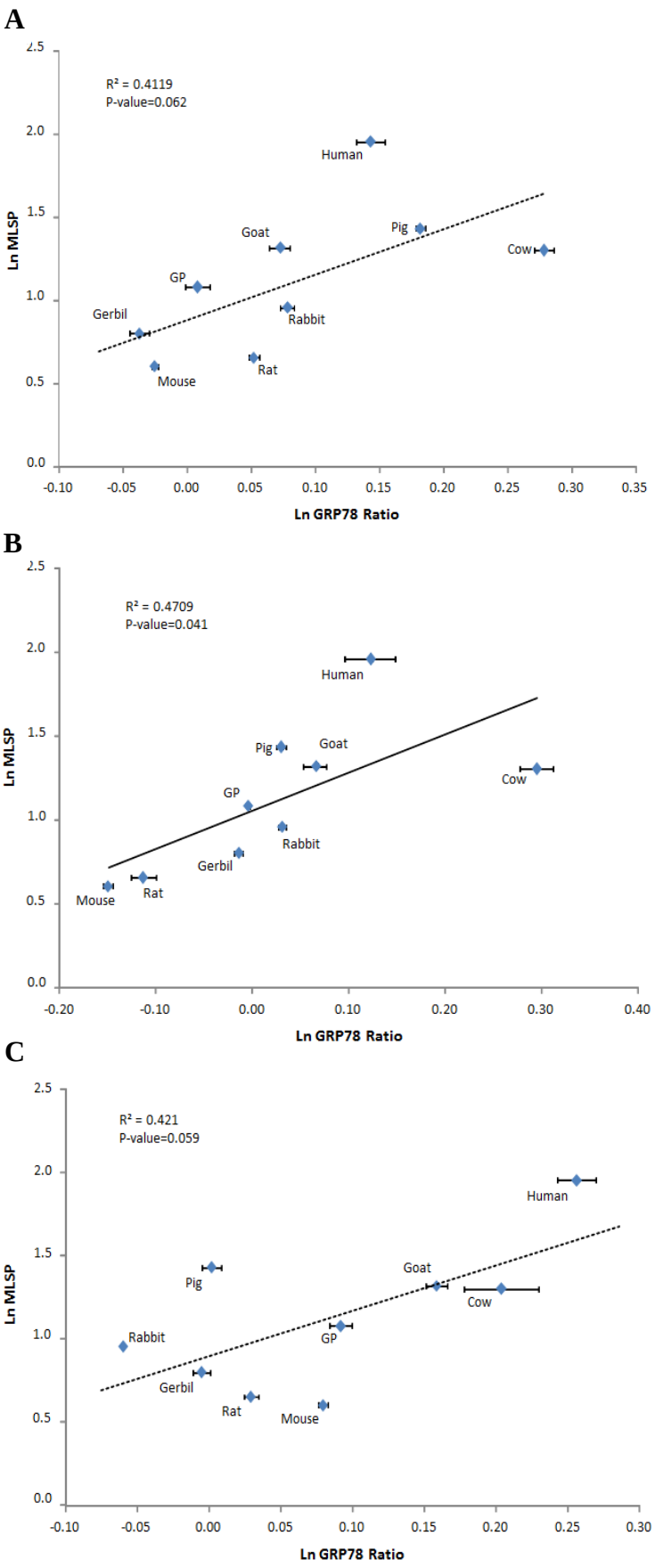

Figure 11: MLSP as a function of GRP78 induction following H_2O_2 exposure after standardization to beta actin. A) MLSP is not correlated to GRP78 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to GRP78 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to GRP78 induction after 3 hour recovery from 3 hour H_2O_2 exposure. GRP78 levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the GRP78 levels from the H_2O_2 exposed cells by GRP78 levels in the time matched unexposed cells.

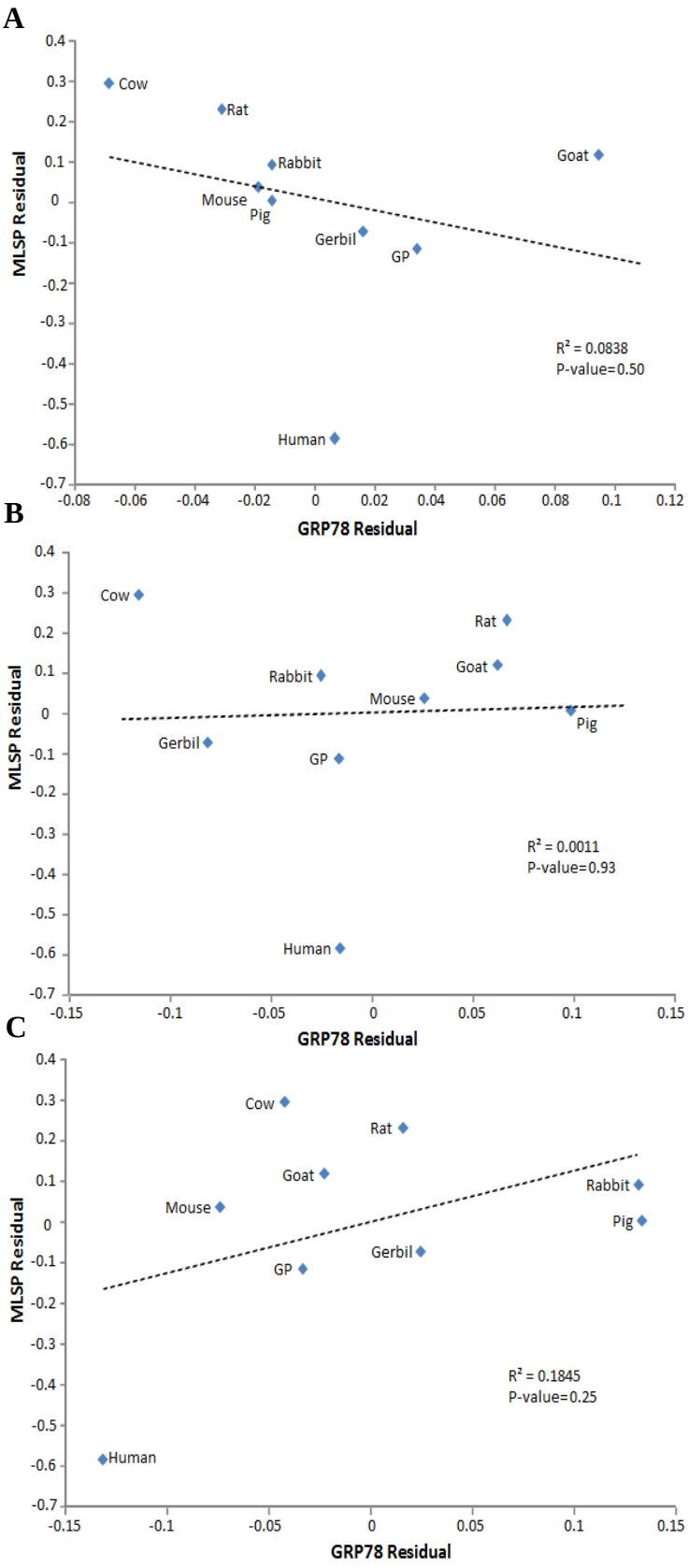

Figure 12: Residual analysis MLSP as a function of GRP78 induction following H_2O_2 exposure after standardization to beta actin. A) MLSP is not correlated to GRP78 induction after 1 hour H_2O_2 exposure. B) MLSP is not correlated to GRP78 induction after 3 hour H_2O_2 exposure. C) MLSP is not correlated to GRP78 induction after 3 hour recovery from 3 hour H_2O_2 exposure. GRP78 levels were measured for 10 mammalian species, with 2-6 individuals per species. All measurements were made in duplicate (two measurements per individual). All inductions were calculated by dividing the GRP78 levels from the H_2O_2 exposed cells by GRP78 levels in the time matched unexposed cells.

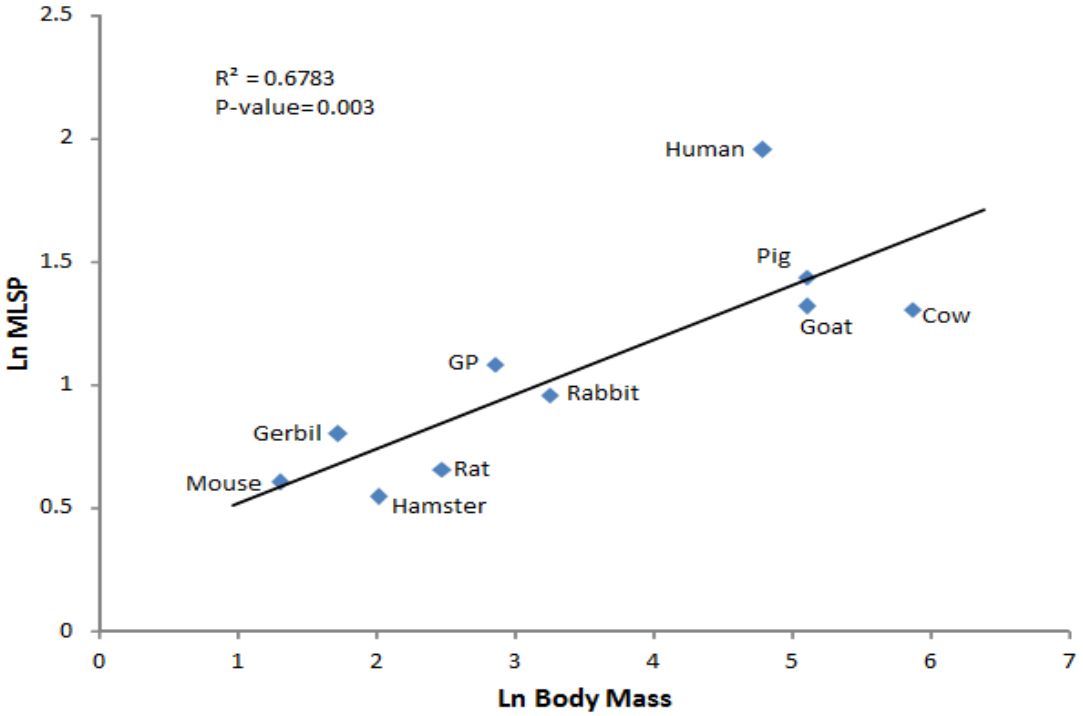

Figure 13: MLSP as a function of body mass for all mammalian species used in this study. MLSP is strongly correlated with mammalian body mass. Species weights and MLSP data are from AnAge (de Magalhaes et al., 2005). Residuals were calculated from this relationship and used in all other residual analysis.